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14. ABSTRACT

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among women in the US. Because metastatic disease is the major cause of death, it is crucial to understand the mechanism by which tumor cells metastasize to the distant organs so that we can identify a better therapeutic target. During this funding period, we found that the metastasis suppressor gene, Drg-1, was capable of down-regulating the ATF3 gene and thus suppressing metastases. We also found that Drg-1 is a good prognostic marker for patient outcome. Our results also suggest that the ATF3 pathway is a potential therapeutic target for patients with metastatic disease. We will focus our next year's effort on further clarification of the Drg-1 pathway and its relation to PTEN.

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INTRODUCTION

Once breast cancer is diagnosed, the most critical question is whether the disease is localized or has it already metastasized to other organs (1). However, the molecular basis of tumor metastasis is poorly understood as yet. The proposed research in this application aims at elucidating the function of the tumor metastasis suppressor gene, Drg-1/NDRG1, in the hope that we can define a specific target for novel and effective therapies to prevent metastatic disease of breast cancer. We hypothesize that NDRG1 functions as a tumor metastasis suppressor in breast cancer (Task 1). We also hypothesize that loss of tumor suppressor PTEN down-regulates NDRG1 gene which leads to metastases (Task 2). We also plan to assess NDRG1 as a diagnostic or prognostic marker to accurately predict metastatic disease. Our ultimate goal is to develop a novel therapeutic method which mimics the function of the NDRG1 gene. We believe that the knowledge gained from the proposed study will eventually be translated into clinical trials.

BODY

Task 1-a.

To examine the effect of NDRG1 on tumor metastases in nude mouse model by injecting NDRG1 expressing cells orthotopically as well as intravenously. We will also examine the expression of the metalloprotease genes at the tumor site in the animals.

We have constructed cell lines that over-express NDRG1 using human breast cell line, MCF7. The expression of the NDRG1 gene was confirmed by PCR and Western blot. We are planning to inject the clones to mice in the near future. The expression of metalloprotease genes, MMP2 and MMP9 were examined on these clones (Fig. 1). However, our preliminary data indicate that NDRG1 did not activate these metalloproteases. We are currently confirming our results by Zymography.

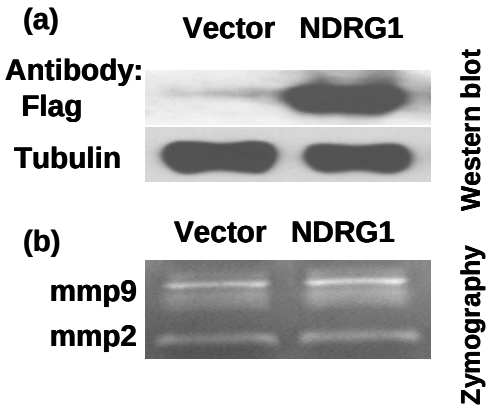


Fig. 1. Expression of NDRG1 does not affect MMP2 or MMP9. (a) NDRG1 was ectopically expressed in MCF7 and cell lysates were prepared. The lysates were then subjected to Western blot (a) and Zymography assays.

Task1-b.

To perform metastasis specific microarray analysis using an inducible NDRG1 expression system to understand downstream effectors of Drg-1.

We have established tetracycline-inducible expression of NDRG1 in a tumor cell and performed a microarray analysis using the Affymetrix human gene array. The results of our microarray analyses indicated that the ATF3 gene, a member of ATF/CREB transcription factor family (2, 3), was most significantly suppressed by induction of the NDRG1 gene. To verify the result of the microarray data, the NDRG1 expression plasmid (pcDNA3/NDRG1) or the empty pcDNA3 vector was transiently transfected into the breast cancer (MCF-7 and MDA-435) as well as prostate (PC3 and ALVA) cell

lines and the level of ATF3 protein was examined by Western blot (Fig. 2 A). We found that NDRG1 indeed attenuated the ATF3 expression in a dose-dependent manner in all these cell lines, while the empty vector did not have any notable effect. In a complementary approach, we introduced NDRG1 siRNA or GFP siRNA in the cancer cells and found that the NDRG1 siRNA specifically abrogated the expression of the NDRG1 gene which led to concomitant up-regulation of the ATF3 expression in these cells (Fig. 2B). These data strongly suggest that NDRG1 plays a crucial role in the in regulation of the ATF*3* gene, and down regulation of Drg-1 in tumor cells results in augmentation of ATF3 expression. To further examine whether down-regulation of ATF3 expression by NDRG1 is mediated at the transcriptional level, tumor cells were co-transfected with NDRG1 expression vector (pcDNA3/NDRG1) or an empty vector (pcDNA3) and ATF3-CAT reporter plasmid, and the CAT reporter assay was performed. We found that the ATF3-CAT reporter activity was significantly attenuated by NDRG1, thereby strongly suggesting that NDRG1 negatively controls the expression of the ATF3 gene at the transcriptional level (Fig. 2C).

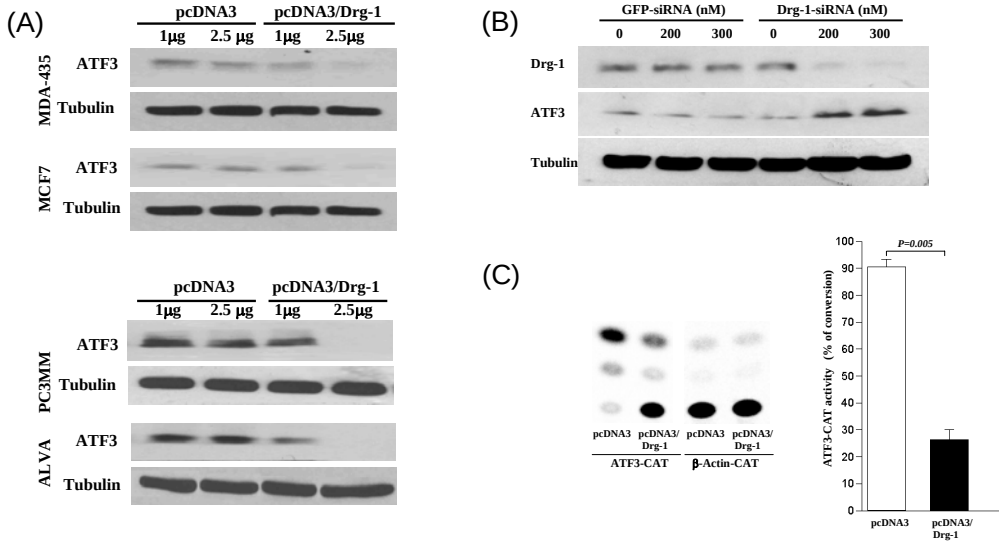


Fig. 2. NDRG1 down-regulates ATF3 expression.
(A), Empty vector pcDNA3 or NDRG1 expression vector, pcDNA3/NDRG1, at the indicated amounts, was transfected into the breast cancer cell lines (MDA-435 and MCF7) and prostate cancer cell lines (PC3MM and ALVA). Forty-eight hour post-transfection, cells were lysed and Western blot was performed using antibodies against ATF3 and Tubulin. (B), siRNA for Drg-1 or GFP was synthesized and various amounts of the siRNA, as indicated, were transfected into PC3MM cells. After 72 hours, cells were lysed and the lysates were examined by Western blot with antibodies for NDRG1, ATF3 and Tubulin. (C), A CAT-reporter plasmid (ATF3-CAT) containing the ATF3 promoter region (-1850 to +34) was co-transfected with NDRG1 expression plasmid (pcDNA3/NDRG1) or empty vector (pcDNA3) into the cells. Forty eight hours later, the cells were harvested, lysed and the lysates were then assayed for the CAT activity. Acetylated chloramphenicol was resolved on TLC plate (representative run, left panel) and each spot was quantified (right panel). A reporter plasmid containing the β-actin promoter (βactin-CAT) was used as a control.

To corroborate the above in vitro results, we established a permanent cell line which expressed the ATF3 gene and they were then injected to SCID mice. The growth of primary tumor was measured for a period of 3 weeks and mice were then sacrificed to examine the metastatic lesions in the lungs. We found that growth rate of primary tumor did not change notably between the tumors with and without

expressing ATF3. However, the number of metastatic lesions in the lungs were significantly increased in the mice that received tumor cells over-expressing ATF3, suggesting that ATF3 indeed is capable of promoting the tumor metastases.

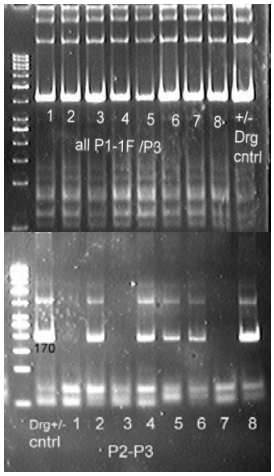
Task 2-a.
To identify the PTEN responsive region on NDRG1 promoter and the factors responsible for the activation

The reporter plasmid of NDRG1 was successfully constructed and we also generated systematic deletion mutants of the promoter region of the NDRG1. The PTEN expression vector is in our hand. We are now conducting a series of experiments to determine the location of PTEN responding region on the promoter of NDRG1 gene. We expect that we will obtain the results of this experiment shortly. Once we identify the region, we will then introduce site-specific mutations to validate the results of deletion analysis.

Task 2-b.
To examine whether the down-regulation of NDRG1 by PTEN indeed leads to metastasis in an animal model

We have not yet pursued this Task at this point extensively. However, we have recently obtained the Drg-1 knockout mouse from Japan and we are establishing a colony of this knockout mouse at SIU (Fig. 3). These mice will be used for this task in the future.

(a)



(b)

	P1/P3	P2/P3
+/+	2kb	170bp
+/-	2kb/400bp	170bp
-/-	400bp	—

(c)

#	Tag		Genotype
1	1179	♂	-/-
2	1180	♂	+/-
3	1181	♂	-/-
4	1182	♂	+/-
5	1183	♂	+/-
6	1184	♀	+/-
7	1185	♀	-/-
8	1186	♀	+/-

Fig. 3. Establishment of an NDRG1 knockout mouse colony. After 3 cycles of backcross breeding, the first generation of homozygous knockout animal was obtained. (a): Results of genotyping by PCR analysis, (b): Expected size of PCR product for homozygous and heterozygous knockout mice, (c): Summary of the first generation of NDRG1 knockout mouse colony.

Task 3-a.
To examine paired samples of primary and lymph node metastases of breast cancer patients for the expression of the NDRG1 gene.

Our immunohistochemical analysis on breast tumor samples indicates that the expression of NDRG1 is strongly down-regulated in the metastatic lesions compared to primary tumors as we expected.

However, the number of samples is still too low to conduct a statistical analysis. We expect to accumulate more than 50 such samples by the end of the 3rd year.

Task 3-b.

The relationship between the expression of the NDRG1 gene and recurrence of the disease will be examined retrospectively in patients over a 10 year period

Our Kaplan-Meier analysis on 85 patients of breast cancer for a period of 5 years indicate that patients with NDRG1 positive expression had significantly more favorable prognosis than those with reduced expression of the gene (P=0.002, log rank test, Fig. 4). Thus, the reduced expression of NDRG1 can be a strong predicator of lymph node and bone metastasis and, in turn, of survival. In multivariate Cox regression analysis involving NDRG1 expression status, primary tumor size and metastasis status, NDRG1 emerged as an independent statistically significant prognostic factor (Table 1). The odds ratio for NDRG1 is 2.435 (95%CI 1.030-5.760, P=0.043), implying that the death risk of patients with reduced NDRG1 expression within a specific time was 2.4 times higher than the risk of patients to die within the same time course with NDRG1 positivity.

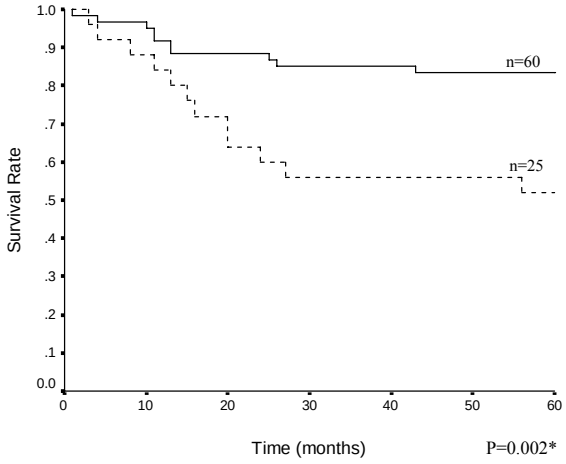


Fig. 4. NDRG1 expression is correlated with survival rate in breast cancer. Disease-free survival rate over a period of 5 years was analyzed in 85 patients in relation to NDRG1 expression. Solid line and dotted line indicate Drg-1 positive patients and patients with reduced expression of NDRG1, respectively. P value was determined by log rank test.

Table 1. **Multivariate Cox regression analysis**

Variables	reference level	b	SE	Wald's x^2	Hazard ratio	95% CI	P
Drg-1	**positive**	**0.890**	**0.439**	**4.107**	**2.435**	**1.030 – 5.760**	**0.043***
Tumor status	**$T_{1-2}N_xM_x$**			**2.264**			**0.132**
Metatsases	**$T_xN_0M_0$**	**1.513**	**0.760**	**3.963**	**4.538**	**1.024 – 20.117**	**0.046***

Thus, the reduced expression of NDRG1 can be a strong predicator of lymph node and bone metastasis and, in turn, of survival. Therefore, these data underscores the clinical relevance of this gene in advancement of breast cancer.

Task 3-c.

To evaluate the status of PTEN, and NDRG1 expression and their relation to survival of breast cancer patients

We have so far performed an immunohistochemical analysis on an archive of 12 breast cancer tissue samples for which we have 5-year's survival data. The results showed that NDRG1 was expressed strongly in the epithelial cells of normal ducts and glands in breast tissue sections, while the poorly differentiated tumor cells in the same specimen had significantly reduced level of NDRG1 (Fig. 5). We also found that the expression of PTEN followed a pattern similar to that of Drg-1, which strongly support our working hypothesis. Although the number of processed samples is too small to draw any conclusions, we observed that patients who have positive staining of both Drg-1 and PTEN tend to have better prognosis. We will continue to process more number of samples this year.

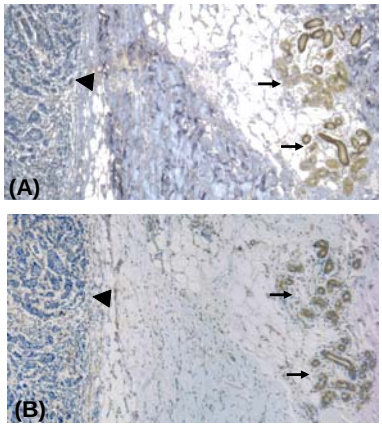


Fig.5. Immunohistochemical analysis of Drg-1 with respect to PTEN and other clinico-pathological parameters in human breast cancer. **(A)** Immunohistochemistry for Drg-1 and PTEN was performed on paraffin tissue sections. A representative field from a breast cancer specimen immunostained with NDRG1 (A) and PTEN (B) antibodies.

KEY RESEARCH ACCOMPLISHMENTS

1. We have identified the ATF3 gene as a down-stream target of NDRG1 by microarray analysis. This result was verified in vitro by over-expression as well as siRNA knockdown of the NDRG1 gene.
2. We found that ATF3 is indeed capable of promoting tumor metastasis without affecting primary tumor growth in an animal model.
3. We have examined the expression of NDRG1 in tumor tissues from breast cancer patients and found that the expression of NDRG1 is inversely correlated with 5-year survival of patients and that NDRG1 can be a strong predicator of lymph node and bone metastasis and, in turn, of survival.
4. The expression of both PTEN and NDRG1 has strong correlation with patient survival.

REPORTABLE OUTCOMES

Peer reviewed publications

1. Bandyopadhyay, S., Zhan, R., Wang, Y., Pai, SK., Hirota, S., Hosobe, S., Takano, Y., Saito, K., Furuta, E., Iiizumi, M., Mohinta, S., Watabe, M., Chalfant, C., and Watabe, K. (2006) Mechanism of apoptosis induced by the inhibition of Fatty Acid Synthase in breast cancer cells. Cancer Res. 66, 5934
2. Furuta, E., Bandyopadhyay, S., Iiizumi,M., Mohinta,S., Zhan,R and Watabe,K. (2006) The role of metastasis suppressors in cancers of breast and prostate. Frontier in Bioscience. 11, 2845-2860
3. Sucharita Bandyopadhyay, Rui Zhan, Asok Chaudhuri, Misako Watabe, Sudha K Pai, Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Yukio Takano, Ken Saito, Mary E Pauza,

Sunao Hayashi, Ying Wang, Sonia Mohinta, Tomoyuki Mashimo, Megumi Iiizumi, Eiji Furuta and Kounosuke Watabe. (2006) Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. Nature Medicine, 12: 933-9388

4. Sucharita Bandyopadhyay, Ying Wang, Rui Zhan, Sudha K Pai, Misako Watabe, Megumi Iiizumi, Eiji Furuta, Sonia Mohinta, Wen Liu, Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Yukio Takano, Ken Saito, Therese Commes, David Piquemal, Tsonwin Hai, and Kounosuke Watabe. (2006) The tumor metastasis suppressor gene Drg-1 down regulates the expression of ATF3 in prostate cancer. *Cancer Res.* 66(24):11983-90.
5. Bandyopadhyay, S and Watabe, K. The Tumor Metastasis Suppressor Gene Drg-1 in Cancer Progression and metastasis in "New developments in Metastasis Suppressors" Ed. By Paul Jackson. Nova Publishers. pp87-102

Abstract/presentation

1. Iiizumi M, Bandyopadhyay S, Hirota, S, Hosobe, S, Tsukada T, Miura K, Saito K, Watabe M, Furuta E, Zhan R, Pai S, Mohinta S and Watabe K. (Apr. 2006) Expression of RhoC correlates with metastatic disease and survival of prostate cancer patients American Association for Cancer Research. Washington DC
2. Bandyopadhyay, S., Zhan, Z., Wang, Y., Pai, SK., Hirota, S., Hosobe, S., Takano, Y., Saito, K., Furuta, E., Iiizumi, M., Mohinta, M., Watabe, M. and Watabe, K. (Apr. 2006) Mechanism of apoptosis induced by the inhibition of Fatty Acid Synthase in breast cancer cells American Association for Cancer Research. Washington DC Apr. 2006
3. Watabe, K. (Oct. 2006) The role of KAI1 and Drg1 in metastases suppression. BenMay Institute Symposium. University of Chicago, Chicago IL.

Employment

1. Dr. Megumi Iiizumi (Postdoc) has been supported by the current grant.
2. Dr. Eiji Furuta (Postdoc) has been partly supported by the current grant.

CONCLUSIONS

During this 2nd year funding period, our major effort was focused on Task 1b and Task3b. We also obtained promising preliminary results in other tasks. Our finding in Task 1b indicates that ATF3 is the target of NDRG1 and ATF3 indeed promotes metastases in an animal model, suggesting that ATF3 and NDRG1 serve as prognostic markers and therapeutic targets for metastatic disease. Because ATF3 is a transcription factor, further down-stream target is of paramount interest. We are currently trying to screen potential targets by promoter scanning.

So what?

Metastatic disease remains the primary cause of death for breast cancer patients. Therefore, it is crucial to identify specific target molecules for better treatment of the patients. Our finding suggests that NDRG1 suppresses tumor metastases by blocking ATF3. Therefore, NDRG1 and ATF3 can be used for diagnostic/prognostic markers as well as for therapeutic targets. Further understanding of the mechanism of NDRG1 function may reveal more rationale targets for the treatment of metastatic disease.

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Mechanism of Apoptosis Induced by the Inhibition of Fatty Acid Synthase in Breast Cancer Cells

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Abstract

Fatty acid synthase (FAS) has been found to be overexpressed in a wide range of epithelial tumors, including breast cancer. Pharmacologic inhibitors of FAS cause apoptosis of breast cancer cells and result in decreased tumor size *in vivo*. However, how the inhibition of FAS induces apoptosis in tumor cells remains largely unknown. To understand the apoptotic pathway resulting from direct inhibition of FAS, we treated breast tumor cells with or without FAS small interfering RNA (siRNA) followed by a microarray analysis. Our results indicated that the proapoptotic genes *BNIP3, tumor necrosis factor–related apoptosis-inducing ligand* (*TRAIL*), and *death-associated protein kinase 2* (*DAPK2*) were significantly up-regulated on direct inhibition of the *FAS* gene. We also found that the knockdown of FAS expression significantly increased ceramide level in the tumor cells, and this increase was abrogated by acetyl-CoA carboxylase inhibitor. In addition, carnitine palmitoyltransferase-1 (CPT-1) inhibitor up-regulated the ceramide and BNIP3 levels in these cells, whereas treatment of tumor cells with FAS siRNA in the presence of a ceramide synthase inhibitor abrogated the up-regulation of BNIP3 and inhibited apoptosis. Furthermore, we found that treatment of cells with BNIP3 siRNA significantly counteracted the effect of FAS siRNA-mediated apoptosis. Consistent with these results, a significant inverse correlation was observed in the expression of FAS and BNIP3 in clinical samples of human breast cancer. Collectively, our results indicate that inhibition of FAS in breast cancer cells causes accumulation of malonyl-CoA, which leads to inhibition of CPT-1 and up-regulation of ceramide and induction of the proapoptotic genes *BNIP3, TRAIL*, and *DAPK2*, resulting in apoptosis. (Cancer Res 2006; 66(11): 5934-40)

Introduction

Mammalian fatty acid synthase (FAS) is a complex multifunctional enzyme that contains seven catalytic domains and a phosphopantotheine prosthetic group on a single polypeptide and catalyzes the synthesis of palmitate from the substrates acetyl-CoA, malonyl-CoA, and NADPH (1). This enzyme also plays a pivotal role in energy homeostasis by converting excess carbon intake into fatty acids for storage, which, when necessary, provide energy via β-oxidation (1). The endogenous synthesis of fatty acid is usually minimal in cells because diet supplies most of the fatty acids, and, consequently, FAS is expressed at low or undetectable level in most normal human tissues, with the exception of lactating breast and cycling endometrium (1). In contrast, FAS is specifically overexpressed in a variety of human malignancies and therefore is considered as an ideal therapeutic target (1–4). For breast cancer, FAS has been reported to be overexpressed in tumor cells, correlate with peritumoral lymphatic vessel invasion and inversely correlate with disease-free survival (5–7). Moreover, treatment of tumor cells with pharmacologic inhibitors of FAS leads to cell growth arrest and apoptosis of breast tumor cells both *in vitro* and *in vivo*, indicating that the elevated level of FAS observed in tumor tissue actually reflects a causal role of the enzyme in tumorigenesis (8–10). However, how up-regulation of FAS promotes tumorigenesis and how inhibition of FAS leads to apoptosis in tumor cells remain unknown, although several possibilities have been speculated. It has been suggested that cell death resulting from the blockade of FAS may be metabolic in origin and occurs due to inhibition of fatty acid β-oxidation (10). Furthermore, malonyl-CoA, which accumulates after treatment of tumor cells with FAS inhibitors, has been implicated, at least in part, in mediating the cytotoxicity (10, 11). However, how the supraphysiologic level of malonyl-CoA leads to apoptosis is not yet known. In this report, we explored the mechanism of induction of apoptosis resulting from direct and specific inhibition of the *FAS* gene by small interfering RNA (siRNA). Our results indicate that apoptosis due to inhibition of FAS in breast tumor cells is mediated by up-regulation of ceramide following induction of the proapoptotic genes *BNIP3, tumor necrosis factor (TNF)–related apoptosis-inducing ligand* (*TRAIL*), and *death-associated protein kinase 2* (*DAPK2*).

Materials and Methods

Cell culture and reagents. Human breast carcinoma cell lines MCF-7, MDA-MB-231, and MDA-MB-435 were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 supplemented with 10% FCS, 100 μg/mL streptomycin, 100 units/mL penicillin, and 250 nmol/L dexamethasone (Sigma Chemical Co., St. Louis, MO) and grown at 37°C in a 5% CO_2 atmosphere. 5-(Tetradecyloxy)-2-furoic acid (TOFA; acetyl-CoA carboxylase inhibitor), fumonisin B_1 (ceramide synthase inhibitor), etomoxir [carnitine palmitoyltransferase-1 (CPT-1) inhibitor], and C_2-ceramide were purchased from Sigma.

siRNA transfection. Four individual siRNAs against the *FAS* gene were designed (sense strand sequences: GAGCGUAUCUGUGAGAAAC, GACGAGAGCACCUUUGAUG, UGACAUCGUCCAUUCGUUU, and CCAUGGAGCGUAUCUGUGA) and custom synthesized by Dharmacon, Inc. (Lafayette, CO). A pool of four species of siRNA against the *BNIP3* gene was also available from Dharmacon (SMARTpool). One siRNA duplex targeting the *green florescent protein* (*GFP*) gene was used as a negative control in all the

Note: S. Bandyopadhyay and R. Zhan contributed equally to this work.

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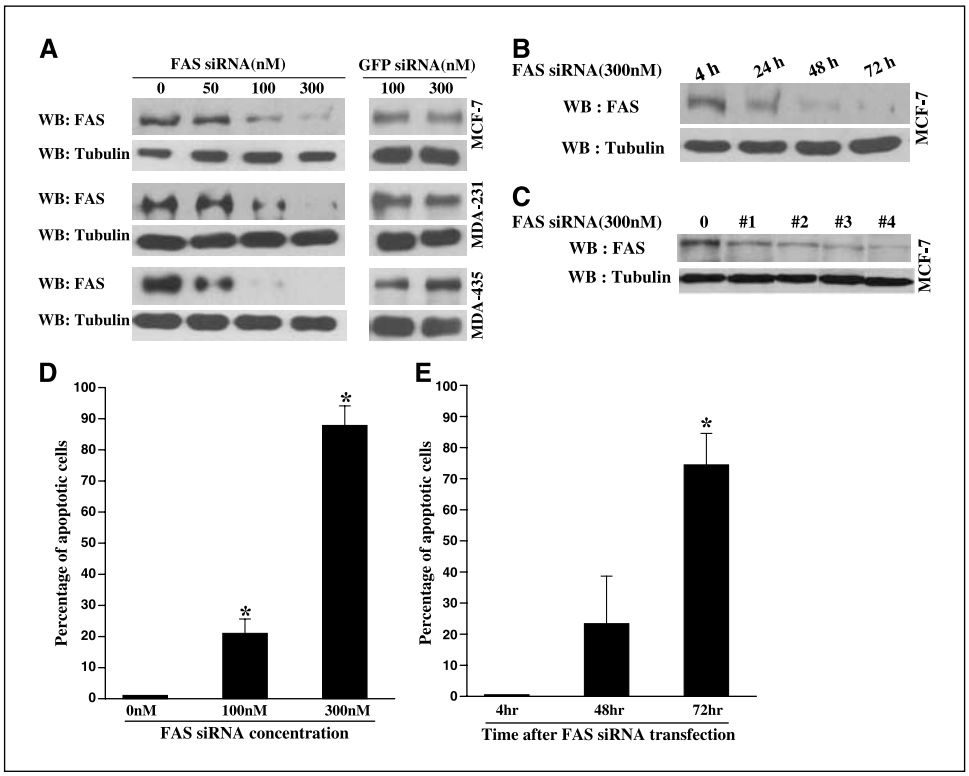


Figure 1. FAS siRNA inhibits expression of the *FAS* gene in breast cancer cells leading to apoptosis. *A,* four individual siRNAs against the *FAS* gene were combined into one pool, and the human breast cancer cell lines MCF-7, MDA-435, and MDA-231 were transfected with various amounts of the pooled FAS siRNA as indicated. *Right,* as a negative control, the cells were also transfected with GFP siRNA. Seventy-two hours after transfection, cells were collected, and the expressions of FAS and tubulin were examined by Western blot (*WB*). *B,* human breast cancer cell line MCF-7 was transfected with 300 nmol/L pooled FAS siRNA and the cells were collected at different time points after transfection as indicated and Western blot analysis for FAS and tubulin was done. *C,* MCF-7 cells were mock transfected (0) or transfected with 300 nmol/L individual FAS siRNA of the pool (*#1-#4*). The cells were collected after 72 hours, and the levels of FAS and tubulin were examined by Western blot. *D* to *E,* MCF-7 cells were transfected with three different doses (*D*) or 300 nmol/L (*E*) FAS siRNA and incubated for 72 hours (*D*) or for various lengths of time as indicated (*E*). The cells were then fixed and permeabilized, and TUNEL assay was done using the *in situ* cell death detection kit/TMR red. The percentage of apoptotic cells in each well was counted under confocal microscope. *, $P < 0.05$, statistically significant correlation.

experiments. The siRNA was transfected into the breast cancer cells using the trans-TKO transfection reagent (Mirus Corp., Madison, WI) according to the manufacturer's protocol.

Western blot. The cells were collected and resuspended in lysis buffer [50 mmol/L Tris-Cl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA]. The lysates were boiled for 5 minutes, resolved by SDS-PAGE on a 10% polyacrylamide gel, and blotted onto nitrocellulose membrane. The membranes were treated with antibodies against FAS (0.2 μg/mL; Immuno-Biological Laboratories Co., Minneapolis, MN), tubulin (1:1,000; Upstate Biotechnology, Charlottesville, VA), BNIP3 (1:500; BioCarta, San Diego, CA), TRAIL (1:500; Stratagene, La Jolla, CA), and DAPK2 (1:500; Chemicon, Temecula, CA). The membranes were then incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies and visualized by enhanced chemiluminescence Plus system (Amersham Life Sciences, Piscataway, NJ).

***In situ* apoptosis assay.** The cells were fixed with 4% paraformaldehyde in PBS followed by permeabilization with 0.2% Triton X-100/0.1% sodium citrate at 4°C. The cells were then washed extensively, and the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was done using the *in situ* cell death detection kit/TMR red (Roche Applied Science, Indianapolis, IN). The reaction was stopped after 1 hour, and the percentage of apoptotic cells in each well was counted under confocal microscope.

Microarray analysis. The cells were collected, and total RNA was prepared using Qiagen (Valencia, CA) RNA isolation kit. The RNA was converted to cDNA and biotinylated followed by hybridization to a human apoptosis and cell cycle-specific cDNA microarray (HS-603, SuperArray Bioscience Corp., Frederick, MD).

Real-time reverse transcription-PCR. Seventy two hours after transfection of the siRNA, total RNA was isolated from the cells and reverse transcribed. The cDNA was then amplified with a pair of forward and reverse primers for the following genes: *BNIP3* (5′-CCTGGGTAGAACTGCACTTCAGCAAT and 5′-TTCATGACGCTCGTGTTCCTCATGCT), *TRAIL* (5′-AAGAGGTCCTCAGAGAGTAG and 5′-TGGTCCATGTCTATCAAGTG), *DAPK2* (5′-CTTTGATCTCAAGCCAGAAAAC and 5′-CTCGTAGTTCACAATTTCTGGAG), *TRAIL-R3* (5′-AAGTTCCTGCACCATGAC and 5′-CCTACGATGGTGCATGAG), *CD40* (5′-CAGGACAGAAACTGGTGAG and 5′-TAAAGACCAGCACCAAGAG), and *β-actin* (5′-TGAGACCTTCAACACCCCAGCCATG and 5′-CGTAGATGGGCACAGTGTGGGTG). PCRs were done using DNA Engine Opticon2 System (MJ Research, South San Francisco, CA) and the DyNAmo SYBR Green qPCR kit (Finnzyme Corp., Espoo, Finland). The thermal cycling conditions comprised an initial denaturation step at 95°C for 15 minutes followed by 30 cycles of PCR using the following profile: 94°C, 30 seconds; 57°C, 30 seconds; and 72°C, 30 seconds.

Ceramide quantitation assay. The ceramide content of the cells was assayed as described before (12). Briefly, total lipid was extracted from the cells, and the dried lipid was solubilized in 11.5 mmol/L Triton X-100 and 0.5 mmol/L cardiolipin by bath sonication and resuspended in a reaction mixture (180 μL) containing 20 mmol/L MOPS (pH 7.2), 50 mmol/L NaCl, 1 mmol/L DTT, 3 mmol/L $CaCl_2$, 0.2 mmol/L diethylenetriaminepentaacetic acid, and 10 μg purified recombinant human ceramide kinase. The reaction was started by the addition of 11 μL ATP [1 μL [γ-^{32}P]ATP (10 μCi/μL) plus 10 μL 20 mmol/L ATP in 100 mmol/L $MgCl_2$] and continued at 37°C for 20 minutes. The reaction was terminated by extraction of lipids with 1.2 mL chloroform/methanol (1:1) and 0.3 mL of 1 mol/L NaCl (13). The organic phase was washed twice with 500 μL of 1 mol/L KCl in 20 mmol/L MOPS. Following extraction of the organic phase, ceramide 1-phosphate was resolved by TLC using ch1oroform/methanol/acetic acid (65:15:5, v/v/v) as solvent and visualized by autoradiography (14).

Immunohistochemistry. Human breast cancer specimens were obtained from surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan). All of the tissue sections were obtained by surgical resection. For immunohistochemical staining, 4-μm-thick sections were cut out from the formaldehyde-fixed and paraffin-embedded tissue specimens and mounted on charged glass slides. The sections were baked at 60°C for 1 hour, deparaffinized by two changes of xylene, and rehydrated in graded alcohol solutions. For antigen retrieval, the sections were either heated in 25 mmol/L sodium citrate (pH 9) at 85°C for 30 minutes (for FAS) or autoclaved in 10 mmol/L sodium citrate buffer (pH 6) for 10 minutes (for BNIP3). The slides were treated with 3% H_2O_2 to block endogenous peroxidase activity and then incubated overnight at 4°C with anti-FAS rabbit polyclonal antibody (1 μg/mL; Immuno-Biological Laboratories) or

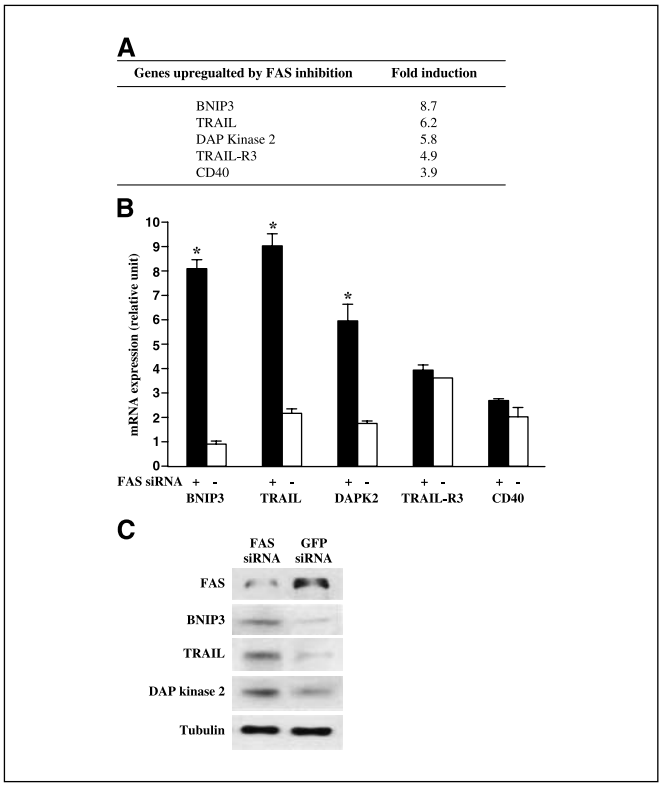


Figure 2. Direct inhibition of the *FAS* gene by siRNA induces the expression of proapoptotic genes in breast tumor cells. *A,* MCF-7 cells were treated with 300 nmol/L FAS siRNA or GFP siRNA for 72 hours, and RNA was analyzed using a human apoptosis and cell cycle-specific microarray. The genes that were most strongly up-regulated as a result of FAS inhibition are listed along with the fold of induction. *B,* MCF-7 cells were treated with FAS siRNA (*black columns*) or GFP siRNA (*white columns*) as described in (*A*). The RNAs were extracted and subjected to real-time quantitative RT-PCR for the five genes as indicated. *, $P < 0.05$, statistically significant correlation. *C,* MCF-7 cells were transfected with the FAS siRNA or GFP siRNA as described above. The cells were then collected and lysed, and the expression of FAS, BNIP3, TRAIL, DAPK2, and tubulin was examined by Western blot.

anti-BNIP3 rabbit polyclonal antibody (1:1,000; BioCarta). The sections were then incubated with HRP-conjugated anti-rabbit IgG for 30 minutes at room temperature, and 3,3′-diaminobenzidine substrate chromogen solution (EnVision Plus kit, Dako Corp., Carpinteria, CA) was applied. Finally, the sections were counterstained with hematoxylin. Results of the immunohistochemistry were judged based on the intensity of staining, comparing between the tumor cells and the normal glands on the same slide. Grading of the FAS and BNIP3 expression level was done by two independent persons without prior knowledge of the patient data. The cases were then divided into those that showed positive staining and those that showed reduced expression of the two genes.

Statistical analysis. For *in vitro* experiments, paired Student's *t* test or one-way ANOVA with Tukey's *post hoc* test was used to calculate *P*s, and in each case, the result represents mean ± SE of three independent experiments. Descriptive statistics comparing between the expression of FAS and BNIP3 were analyzed by standard χ^2 test. For all of the statistical tests, the significance was defined as $P < 0.05$. In all cases, Statistical Package for the Social Sciences software was used.

Results

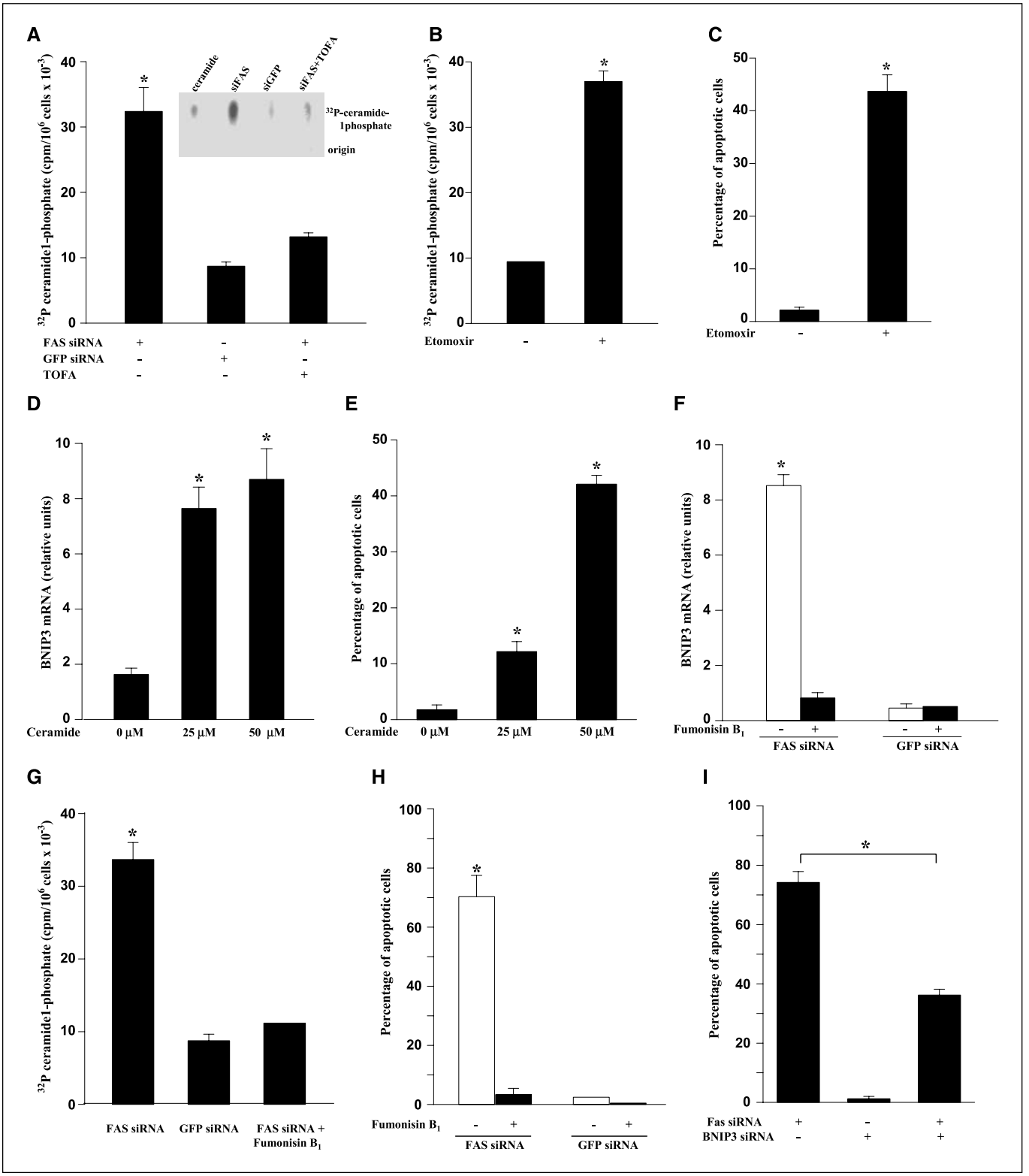
Specific inhibition of the *FAS* gene induces apoptosis in breast tumor cells *in vitro*. To examine the effect of direct inhibition of the expression of the *FAS* gene in breast cancer cells, a pool of four individual siRNAs against the *FAS* gene (FAS siRNA) was transfected into FAS-positive breast cancer cell lines MCF-7, MDA-231, and MDA-435. As shown in Fig. 1*A*, in each of the tested cell lines, the FAS siRNA inhibited expression of the *FAS* gene in a dose-dependent manner, whereas the GFP siRNA had no appreciable effect, indicating the specific effect of this set of siRNA in knocking down the expression of the *FAS* gene in human breast tumor cells. We also observed the time-dependent nature of this inhibition, with a significant effect being noted after 48 hours (Fig. 1*B*). Furthermore, as shown in Fig. 1*C*, each of the individual siRNA species within the pool was also able to knock down the expression of the *FAS* gene albeit with less efficiency. Next, to examine the end result of the direct inhibition of the *FAS* gene, MCF-7 cells were transfected with various amounts of FAS siRNA, and the extent of apoptosis was measured by assessing DNA fragmentation. As shown in Fig. 1*D* to *E*, FAS siRNA significantly augmented the degree of apoptosis in both dose and time-dependent manner ($P < 0.05$), whereas the GFP siRNA did not have any notable effect (data not shown). Our results therefore suggest that these siRNAs, specifically targeted to the *FAS* gene, lead to apoptosis of tumor cells and may have potential therapeutic utility.

Inhibition of FAS leads to up-regulation of proapoptotic genes. To understand the mechanism of apoptosis induced by inhibition of FAS, we treated MCF-7 cells with 300 nmol/L FAS siRNA or GFP siRNA for 72 hours, and their RNA was prepared. These RNAs were converted to cDNA that was then used for hybridization with the apoptosis/cell cycle-specific microarray, which contains 96 key apoptosis genes, 96 key cell cycle regulation genes, and 75 stress and toxicity genes. Among the genes whose expressions were significantly altered by FAS inhibition, the five most strongly up-regulated genes were *BNIP3, TRAIL, DAPK2, TRAIL-R3*, and *CD40* (Fig. 2*A*). To confirm the result of the microarray analysis, we treated the MCF-7 cells with FAS siRNA in a similar manner and did real-time reverse transcription-PCR (RT-PCR) on the RNA samples. As shown in Fig. 2*B*, among these

Figure 3. Apoptosis induced by inhibition of FAS in human breast cancer cells is mediated by ceramide and BNIP3. *A,* MCF-7 cells were transfected with 300 nmol/L FAS siRNA, GFP siRNA, or a combination of FAS siRNA and TOFA as indicated. Seventy-two hours after transfection, the cells were collected and total lipid was extracted and assayed for ceramide using ceramide kinase. *Inset,* autoradiograph of TLC plate for ceramide, where control (*lane 1*) containing authentic ceramide 1-phosphate prepared enzymatically was used to validate the position of the cellular ceramide 1-phosphate. *B* to *C,* MCF-7 cells were treated with 20 μmol/L etomoxir (+) or 70% alcohol (−) for 48 hours, the level of cellular ceramide was assayed as described in (*A*) above (*B*), or the extent of apoptosis was measured by *in situ* apoptosis assay as described in Fig. 1*D* to *E* (*C*). *D* to *E,* MCF-7 cells were treated for 24 hours with various doses of C_2-ceramide as indicated, the expression of *BNIP3* and *β-actin* genes were examined by quantitative RT-PCR (*D*), and the degree of apoptosis was measured by *in situ* apoptosis assay as described in Fig. 1*D* to *E* (*E*). *F,* MCF-7 cells were transfected with FAS siRNA or GFP siRNA as indicated and then treated with (*black columns*) or without (*white columns*) 50 μmol/L of ceramide synthase inhibitor, fumonisin B_1. RNA was extracted from the cells, and the expression of *BNIP3* and *β-actin* genes were examined by real-time RT-PCR. *G,* MCF-7 cells were treated with 300 nmol/L FAS siRNA or GFP siRNA or a combination of FAS siRNA and 50 μmol/L fumonisin B_1, and the level of cellular ceramide was assayed as described in (*A*). *H,* MCF-7 cells were transfected with FAS siRNA or GFP siRNA as indicated and then treated with (*black columns*) or without (*white columns*) 50 μmol/L fumonisin B_1, and the degree of apoptosis was measured by an *in situ* apoptosis assay as described in Fig. 1*D* to *E*. *I,* MCF-7 cells were transfected with 300 nmol/L of FAS siRNA or BNIP3 siRNA or a combination of both as indicated, and 48 hours after transfection, the degree of apoptosis was measured by *in situ* apoptosis assay. *, $P < 0.05$, statistically significant correlation.

genes, the expressions of *BNIP3, TRAIL*, and *DAPK2* were found to be reproducibly up-regulated by FAS siRNA (P = 0.004, 0.009, and 0.029, respectively). In addition, as shown in the result of Western blot analysis (Fig. 2*C*), expressions of these genes were also found to be augmented at the protein level following the FAS inhibition.

Inhibition of FAS leads to up-regulation of ceramide synthesis. Malonyl-CoA, the substrate for FAS, has been known to inhibit CPT-1 (15). On the other hand, inhibition of CPT-1 was previously found to lead to accumulation of ceramide (16). Therefore, to explore a possibility that inhibition of FAS leads to apoptosis via up-regulation of ceramide, we treated the MCF-7



breast tumor cells with 300 nmol/L FAS siRNA or GFP siRNA for 72 hours and measured the level of ceramide in the cells. As shown in Fig. 3*A*, treatment with FAS siRNA resulted in a significant up-regulation of ceramide synthesis in these cells compared with the treatment with GFP siRNA ($P = 0.001$). We observed a similar elevation of ceramide level in MDA-435 breast cancer cells following FAS siRNA treatment (data not shown). To clarify the involvement of malonyl-CoA in up-regulation of ceramide by FAS siRNA, we treated the MCF-7 cells with TOFA, an inhibitor of acetyl-CoA carboxylase that catalyzes synthesis of malonyl-CoA from acetyl-CoA. As shown in Fig. 3*A*, TOFA significantly inhibited ceramide accumulation in the presence of FAS siRNA ($P = 0.002$), suggesting the active role of malonyl-CoA in FAS siRNA-mediated ceramide up-regulation. Because malonyl-CoA is known to inhibit CPT-1, we next explored the effect of etomoxir, an inhibitor of CPT-1, on the MCF-7 cells. As shown in Fig. 3*B*, treatment with etomoxir resulted in a significant accumulation of ceramide in these cells ($P = 0.003$), and the degree of accumulation of ceramide was found to be comparable with that observed by FAS siRNA treatment. Furthermore, as shown in Fig. 3*C*, treatment with etomoxir also induced a substantial degree of apoptosis in the MCF-7 cells ($P < 0.0001$). These results strongly suggest that FAS siRNA leads to accumulation of malonyl-CoA, which in turn leads to inhibition of CPT-1, resulting in accumulation of ceramide.

Because our microarray analysis indicated that FAS inhibition leads to up-regulation of BNIP3 expression, we examined the involvement of ceramide in overexpression of BNIP3 and apoptosis following FAS inhibition. Toward that end, we first tested the effect of exogenously added C_2-ceramide on MCF-7 cells. As shown in Fig. 3*D*, 25 and 50 μmol/L of C_2-ceramide significantly up-regulated the BNIP3 mRNA level in these cells as tested by real-time RT-PCR ($P = 0.004$ and 0.002, respectively). In addition, we observed a significant degree of induction of apoptosis by 25 μmol/L C_2-ceramide ($P < 0.0001$), which was further enhanced by the 50-μmol/L dose ($P < 0.0001$; Fig. 3*E*). To test whether up-regulation of ceramide is an essential step in FAS siRNA-mediated BNIP3 up-regulation, MCF-7 cells were treated with FAS siRNA or GFP siRNA with or without fumonisin B_1 (a ceramide synthase inhibitor), and the level of BNIP3 expression was examined by quantitative RT-PCR. As shown in Fig. 3*F*, FAS siRNA significantly augmented the BNIP3 expression in these cells ($P < 0.0001$), and this effect was nullified by treatment with the ceramide inhibitor, indicating a positive involvement of ceramide in FAS siRNA-mediated up-regulation of BNIP3 expression. A ceramide quantitation assay was also done to confirm that fumonisin B_1 indeed significantly abolished the ceramide level in these cells in the presence of FAS siRNA ($P < 0.0001$; Fig. 3*G*). In addition, we examined the effect of fumonisin B_1 on FAS siRNA-mediated apoptosis and found that the MCF-7 cells underwent apoptosis when treated with FAS siRNA but not with GFP siRNA ($P < 0.0001$) as expected (Fig. 3*H*). However, apoptosis induced by FAS siRNA was significantly inhibited on treatment with fumonisin B_1 ($P < 0.0001$). Furthermore, to corroborate the role of BNIP3 in FAS siRNA-mediated apoptosis, we treated the cells with FAS siRNA, BNIP3 siRNA, or a combination of both and found that BNIP3 siRNA significantly counteracted the effect of FAS siRNA ($P < 0.0001$; Fig. 3*I*). Taken together, these results strongly suggest that FAS siRNA causes apoptosis in breast tumor cells via up-regulation of the *BNIP3* gene primarily through a ceramide-dependent pathway.

Expression of FAS and BNIP3 inversely correlates in human breast cancer. To examine whether our finding that inhibition of FAS leads to BNIP3 up-regulation is also reflected in the clinical setting, we examined the levels of FAS and BNIP3 proteins in a set of breast cancer samples. As shown in Fig. 4*A*, expression of FAS was almost undetectable in normal mammary ducts and glands, whereas the protein was strongly expressed in the poorly differentiated tumor cells in the same patient. On the other hand, BNIP3 was found to be abundantly expressed in the epithelial cells of normal ducts and glands, whereas expression of the protein was significantly reduced in tumor cells. Importantly, as shown in two representative fields in Fig. 4*A*, almost reverse staining pattern was observed when the same field was examined for *FAS* and *BNIP3* expression. Statistical evaluation also revealed a significant inverse correlation between expression status of these two genes ($P = 0.018$, Fig. 4*B*). These results are consistent with our notion that inhibition of FAS leads to up-regulation of the proapoptotic gene *BNIP3* in breast cancer cells and suggest a possibility that FAS protects the breast cancer cells through down-regulation of the *BNIP3* gene.

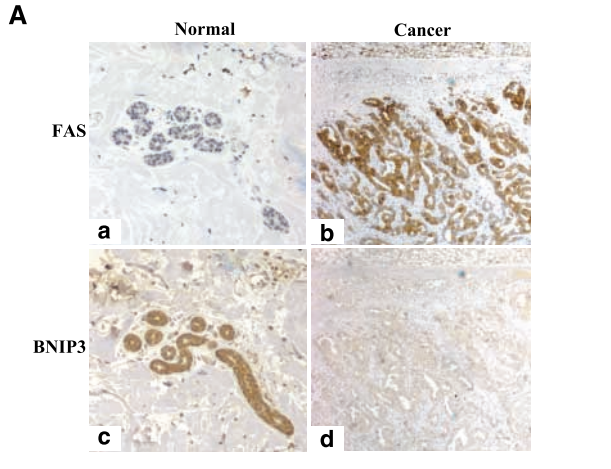
Discussion

FAS is found to be overexpressed in various types of cancers and has been suggested that such up-regulation of FAS provides some selective advantage to the tumor cells by promoting proliferation and inhibiting apoptosis (1). Consistent with this idea, the chemical inhibitors of FAS have been shown to induce apoptosis in cancer cells in culture and decrease tumor size in animal models of various cancers (1). However, how inhibition of FAS promotes apoptosis in tumor cells remains elusive. In this report, to understand the mechanism of apoptosis induced by FAS inhibition, we used FAS siRNA to knock down FAS expression in a specific manner. We first showed the efficacy of the set of FAS siRNA to directly abrogate the expression of the *FAS* gene and trigger apoptosis, which is also consistent with the recent reports from our group and others, where FAS siRNA has been shown to cause apoptosis in various prostate cancer cells *in vitro* (17, 18).

FAS inhibition by the synthetic inhibitors, such as cerulenin or siRNA, has been observed to lead to a significant accumulation of malonyl-CoA in tumor cells, as would be expected from the fact that FAS uses malonyl-CoA as a substrate (10, 11, 19). We have also shown in this report that inhibition of malonyl-CoA synthesis significantly counteracts the effect of FAS siRNA, indicating an important role of malonyl-CoA accumulation in FAS siRNA-mediated apoptosis. Malonyl-CoA is also a physiologic inhibitor of the mitochondrial outer membrane enzyme CPT-1 that transesterifies long-chain acyl-CoAs to acylcarnitine, permitting their entry into the mitochondria for fatty acid oxidation (15). It is therefore plausible that inhibition of FAS that causes malonyl-CoA accumulation may lead to concomitant inhibition of CPT-1. Thupari et al. (10) have indeed observed that inhibition of FAS by cerulenin causes CPT-1 inhibition, and in agreement with this observation, we have found that inhibition of CPT-1 mimics the effect of FAS siRNA. Interestingly, inhibition of CPT-1 has been found to be significantly correlated with accumulation of ceramide, a sphingolipid that has been implicated in apoptotic response of cells to death inducers, such as Fas/Fas ligand, TNF-α, growth factor withdrawal, hypoxia, and DNA damage (16, 20). In this report, we showed that treatment of breast tumor cells with FAS siRNA significantly augmented the synthesis of ceramide and concomitant cell death. Furthermore, we provided evidence that exogenous ceramide mimicked the effect of FAS siRNA in these cells, whereas a ceramide synthase inhibitor significantly

counteracted the effect of FAS siRNA. Taken together, our results strongly indicate that direct inhibition of FAS by siRNA leads to accumulation of malonyl-CoA, which in turn inhibits CPT-1, resulting in up-regulation of ceramide and apoptotic cell death. Because FAS is found to be up-regulated in a variety of cancers, it will be interesting to test whether overexpression of FAS *in vivo* results in tumorigenesis via suppression of ceramide synthesis pathway.

The results of our microarray analysis indicated that FAS-mediated apoptotic pathway involved induction of the proapoptotic genes *BNIP3, TRAIL*, and *DAPK2*. BNIP3 is a mitochondria-associated cell death protein, originally identified as an adenovirus E1B 19-kDa-interacting protein (21). Consistent with the proapoptotic function of *BNIP3*, the gene is found to be significantly down-regulated in various types of cancers, including pancreatic, colorectal, gastric, and hematopoietic cancer cells, and this down-regulation occurs at least in part by hypermethylation of the promoter of the *BNIP3* gene (22–24). BNIP3 not only induces apoptotic cell death but also is implicated in necrosis and autophagy (25, 26). Cell death induced by BNIP3 has been found to be caspase independent but is accompanied by rapid and profound mitochondrial dysfunction (25). Interestingly, C_2-ceramide has been shown to up-regulate the expression of BNIP3, leading to autophagic cell death in malignant glioma cells (26). This result is consistent with our finding of increased ceramide synthesis and BNIP3 up-regulation following FAS inhibition by siRNA. Moreover, our observations that BNIP3 up-regulation and apoptosis induced by FAS siRNA is nullified by the ceramide synthase inhibitor and



B

Expression of BNIP3 and FAS is inversely correlated

	All (17)	FAS expression positive (12)	reduced (5)	P value
BNIP3 status				
Positive	5	1 (20%)	4 (80%)	
Negative	12	11 (92%)	1 (8%)	0.018*

Figure 4. Expression of FAS and BNIP3 is inversely correlated in human breast cancer. *A,* immunohistochemistry for FAS and BNIP3 was done on paraffin-embedded tissue sections from breast cancer patients. FAS immunostaining in a representative field from a sample showing normal mammary ductolobular unit (*a*) and poorly differentiated carcinoma (*b*). *c* to *d,* same fields after immunostaining for BNIP3. *B,* association between FAS and BNIP3 expression in a set of breast cancer cases was analyzed by χ^2 test. *, $P < 0.05$, statistically significant correlation.

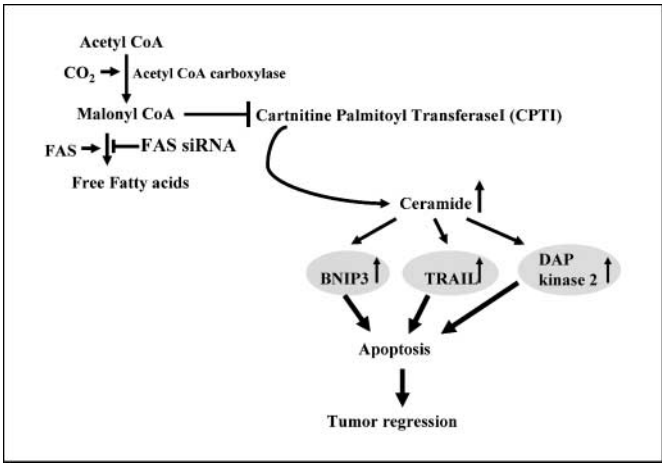


Figure 5. A proposed apoptotic pathway induced by direct inhibition of the *FAS* gene.

that FAS siRNA-mediated apoptosis is significantly abrogated by BNIP3 siRNA strongly suggest that apoptotic cell death resulting from FAS inhibition occurs via up-regulation of ceramide synthesis following BNIP3 overexpression.

TRAIL is a proapoptotic gene of the TNF family that has been shown to induce apoptosis in a wide range of transformed cell lines (27). We found that FAS inhibition by siRNA that led to overexpression of ceramide was also associated with up-regulation of the expression of the *TRAIL* gene. Consistent with our result, Herr et al. (28) showed that C_2-ceramide increased the expression of TRAIL in neuroblastoma cells. Furthermore, recently, in a microarray analysis of a set of prostate cancer patients, Rossi et al. (29) observed a significant inverse correlation between FAS and TRAIL expression, which is in good agreement with our finding that TRAIL is a downstream component of the apoptotic pathway initiated by FAS inhibition. *DAPK2* is a proapoptotic gene encoding a protein that belongs to the serine/threonine protein kinase family (30, 31). This protein contains a NH_2-terminal protein kinase domain followed by a conserved calmodulin-binding domain with significant similarity to that of DAPK1, which also is a positive regulator of programmed cell death (32). Interestingly, DAPK1 activity is critical for the apoptotic cascade involving C_2-ceramide and C_8-ceramide, although the direct involvement of DAPK2 in ceramide-mediated apoptotic pathway remains to be shown (33, 34).

Based on our results, we propose a model for the apoptotic pathway induced by FAS inhibition, whereby inhibition of FAS leads to accumulation of malonyl-CoA, which in turn inhibits CPT-1 resulting in up-regulation of ceramide followed by induction of the proapoptotic genes *BNIP3, TRAIL*, and *DAPK2* (Fig. 5). It will be interesting to test the proposed pathway in an animal model, and understanding this pathway will provide new insights into cancer cell metabolism and aid in designing more specific anticancer drugs.

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The role of tumor metastasis suppressors in cancers of breast and prostate

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1. ABSTRACT

Despite significant improvement in surgical techniques and chemotherapies, none of the current medical technologies “cure” metastatic disease, and the patients who have acquired metastatic cancer inevitably die from disseminated disease. Thus, there is a need for developing novel therapeutic approaches which can directly target metastatic tumor cells. However, advances in understanding the molecular mechanism of tumor metastases have lagged behind other developments in the cancer field. Tumor metastasis involves complex array of steps with each step requiring a coordination of the actions of many positive and negative factors. A number of tumor metastasis suppressors have been identified which suppress the formation of tumor metastasis without affecting the growth rate of the primary tumor. Such discoveries offer new approaches for curtailing tumor metastasis. This review summarizes our current understanding on these genes and their potential role in the progression of tumor metastases.

2. CLINICAL SIGNIFICANCE OF TUMOR METASTASES

Malignant tumors metastasize to adjacent or distant organs through the blood vascular circuit or lymphatic system. When cancer is detected at an early stage, before it has spread to other distant sites, it can be treated successfully by surgery or local irradiation and the patient will be cured. However, treatments are much less successful when the cancer is detected after it has already metastasized. Unfortunately, most patients present with a metastatic disease at the time of the first visit to the clinic, and in addition, many patients who do not present any evidence of metastasis at the time of their initial diagnosis, metastases will be detected at a later time. Therefore metastatic disease is a serious concern for survival of cancer patients. In spite of this clinical importance of metastasis, much remains to be learned about the biology of the metastatic process.

It is well known, based both on clinical observations and mechanistic studies, that metastasis

formation is an inefficient process (1). Although large numbers of tumor cells are shed into the vascular drainage system from a primary tumor, it has been demonstrated experimentally that, after intravenous injection of highly metastatic tumor cells, approximately only 0.01% of these cells form tumor foci (2, 3). The inefficiency of tumor cells in completing the metastatic cascade results from the fact that successful formation of metastatic foci consists of several highly complex and interdependent steps. Each step is rate-limiting in that, failure to complete any of these events totally disrupts metastasis formation (1). The steps involved in metastasis formation are described below.

3. PROCESS OF TUMOR METASTASES

After the initial neoplastic transformation, the tumor cells undergo progressive proliferation that is accompanied by further genetic changes and development of a heterogeneous tumor cell population with varying degrees of metastatic potential. The oncogenic transformation is a result of the balance between the proto-oncogenes, which gain function by mutation, and the tumor suppressor genes, which contribute to tumorigenesis by loss of function (4, 5). The initial growth of the primary tumor is supported by the surrounding tissue microenvironment, which eventually becomes rate-limiting for further growth. As the tumor grows and the central tumor cells become hypoxic, the tumor initiates recruitment of its own blood supply. This process is referred to as the angiogenic switch and involves a balance between secretion of various angiogenic factors and removal or suppression of angiogenesis inhibitors (6, 7). The numerous positive and negative factors involved in angiogenesis are listed in Table 1. Notably, the process of neovascularization is almost invariably associated with a dramatic increase in the metastatic potential of tumors.

Continued genetic alteration in the tumor cell population results in selection of tumor cell clones with distinct growth advantage and acquisition of an invasive phenotype. Invasive tumor cells down-regulate cell-cell adhesion by modulating the expression of cadherins, alter their attachment to the extracellular matrix by changing integrin expression profiles and proteolytically alter the matrix by secretion of the matrix metalloproteases (1). Collectively, these changes result in enhanced cell motility and the ability of these invasive cells to separate from the primary tumor mass. These cells can detach from the primary tumor and create defects in the extra-cellular matrix that define tissue boundaries such as basement membranes, thus accomplishing stromal invasion. Furthermore, the poorly formed tumor vasculature that is generated in response to the angiogenic switch in the primary tumor mass, as well as thin walled lymphatic channels in the surrounding stroma, are readily penetrated by these invasive tumor cells and offer ready conduits to the systemic circulation (6). Endothelial cells responding to the angiogenic stimulus produced by the primary tumor also express an invasive phenotype and greatly enhance the metastatic process (7).

Once the tumor cells and the tumor cell clumps (emboli) have reached the vascular or lymphatic compartments, they must survive a variety of hemodynamic and immunologic challenges. Because cancer cells often express tumor specific antigens, they are attacked by non-specific (macrophage and NK cells) as well as specific (T cells) immune systems. However, some tumor cells evade the immune surveillance by a variety of mechanisms such as down-regulation of MHCI (8) and secretion of Fas ligand (9). After survival in the circulation, tumor cells must arrest in distant organs or lymph nodes. This arrest may occur by size trapping on the inflow side of microcirculation, or by adherence of tumor cells through specific interactions with capillary or lymphatic endothelial cells, or by binding to exposed basement membrane. In most cases, arrested tumor cells extravasate before proliferating. After exiting the vascular or lymphatic compartments, metastatic tumor cells may proliferate in response to paracrine growth factors or become dormant. After extravasation, tumor cells migrate to a local environment more favorable for their continued growth. Findings using *in vivo* video-microscopy demonstrate that the poor growth of tumor cells after extravasation from the circulation is a major factor contributing to the inefficiency of the metastatic process (10).

According to a century-old theory, a disseminated cancer cell acts like a seed, growing only if it finds suitable soil at a secondary site. Support for this idea comes from the observation that the target organ of metastasis is typically better than non-target organs in stimulating the growth of cancer cells *in vitro* (11). For example, researchers have noted that the bone marrow, in contrast to various other organs, strongly stimulates prostate cancer cell growth *in vitro* but has little or no effect on cancer cells that metastasize to non-bone organs (12). Similar correlations have been made for cancer cells *in vivo*. In a study of mammary cancer sublines with varying patterns of metastasis, the preferred organ of metastasis in each case was the organ allowing the most rapid growth of cancer cells (13). A traditional alternative to the "seed and soil" argument, known as the anatomical-mechanical hypothesis, challenges the importance of the soil in regulating cancer cell growth. It argues instead that metastasis develops in the organ of any capillary bed in which a disseminated cancer cell becomes mechanically lodged (11). Consistent with this hypothesis, it was noted in the 1940s that specific veins draining the prostate encountered their first capillary bed in the lumbar spine, which is a common site of prostate cancer metastasis (14). More recent findings also suggest that the cancer cell may have an important role in modifying the environment that it encounters. The environment reacts to this modification by inducing changes in the tumor cell and the cycle repeats (15). Hence, according to this model, the regulatory interaction between seed and soil is dynamic and reciprocal.

4. TUMOR METASTASES SUPPRESSOR GENES AND THEIR ROLES IN CANCER PROGRESSION

As described above, the process of tumor metastases involves multiple steps with high complexity and each step requires a coordination of the actions of

Table 1. Factors involved in the process of tumor metastases

Factor	Function	Expression in cancer	Location	Reference
Positive Factor				
Twist	Transcription, Cell adhesion	Breast, Prostate	7p21.2	109, 110
MMP2	Degrades extracellular matrix	Breast, Lung	16q13-q21	111, 112
MMP7	Degrades extracellular matrix	Colorectal, Gastric, Lung	11q21-q22	113-115
Catenin alpha 1	Cell signaling	Pancreatic	5q31	116
Catenin beta 1	Cell signaling	Breast, Prostate	3p21	117, 118
uPA	Serine protease	Breast, Prostate, Colorectal	10q24	119-121
Reptin	ATPase, DNA helicase activity	Prostate	19q13.3	118
VEGF	Angiogenesis	Breast, Prostate, Colorectal	6p12	121-123
PLGF	Angiogenesis	Breast	14q24-q31	124
FGF 1	Cell proliferation, Angiogenesis	Prostate	5q31	125
FGF 4	Cell proliferation, Angiogenesis	Prostate	11q13.3	125
TGF beta	Cell proliferation, differentiation	Breast, Prostate	19q13.1	126, 127
EGF	Cell proliferation, mitogenicity	Breast, Prostate	4q25	128, 129
PDGF	Embryological development	Breast, Prostate	22q13.1	130, 131
GCSF	Cell growth, Survival	Prostate	17q11.2-q12	132
IL-8	Angiogenesis	Breast, Prostate, Clorectal	4q13-q21	121, 133, 134
Angiogenin	Angiogenesis	Breast, Prostate	14q11.1-q11.2	135, 136
CD44	Cell adhesion, migration	Breast, Prostate	11p13	137
HGF	Cell growth, motility	Breast, Prostate, Lung	7q21.1	138-140
AMF	Glycolysis, Neurotropic factor	Breast, Prostate	19q13.1	141, 142
Snail homolog 2	Transcriptional repressor	Breast, Liver	8q11	143, 144
Negative Factor				
E-cadherin	Cell adhesion	Breast, Prostate, Lung	16q22.1	145-147
Fibronectin 1	Cell adhesion molecule	Breast	2q34	148
Vimentin	Cell adhesion molecule	Prostate	10p13	149
Thrombospondin 1	Angiogenesis	Breast	15q15	150
Angiostatin	Angiogenesis	Breast, Prostate	6q26	151, 152
Endostatin	Angiogenesis	Hepatoma	21q22.3	153
Vasostatin	Angiogenesis	Lung	14q32	154

many positive and negative factors. The fact that fusing a non-metastatic cell with highly metastatic cancer cell results in suppression of metastatic ability of the tumor cell raised a hypothesis that tumor metastasis is negatively regulated by tumor metastasis suppressor genes (16). They are defined as genes that suppress the formation of metastases, without affecting the growth rate of the primary tumor. Search for such genes using multiple approaches such as micro-cell mediated chromosome transfer (MMCT), microarray analyses and subtractive hybridization, has been quite effective, and to date, there are fourteen identified genes that clearly meet this criterion (Table 2). The following section summarizes the current information on each of these genes.

4.1. NM23

NM23 was the first gene isolated as a tumor metastasis suppressor. To identify a differentially expressed gene involved in tumor metastasis, Steeg *et al.* utilized a series of related murine melanoma cell lines of varying metastatic potential (17). By subtractive hybridization between the mRNAs from cell lines with low and high metastatic potential,the NM23 gene was isolated (17). They noted that NM23 mRNA levels did not correlate with cells' sensitivity to host immunological responses and therefore must be associated with intrinsic aggressiveness. In addition to the clinical observation of the down-regulation of NM23 gene expression in breast carcinoma (18), transfection of NM23 into highly metastatic breast, melanoma, colon, and oral squamous cell lines reduced *in vivo* metastatic potential of these cells (19-21). In addition, transfection of human NM23 into human breast carcinoma cells reduced *in vitro* motility to numerous attractants and inhibited colonization in soft agar (19). The metastasis suppressive activity of NM23 was previously correlated with its histidine protein kinase activity although physiological substrates for this unusual kinase activity have not been identified (22). Hartsough *et al.* reported that NM23 co-immunoprecipitated with the KSR (kinase suppressor of Ras) protein and phosphorylated ser-392 and ser-434 on KSR (23). It has been hypothesized that phosphorylation of KSR by NM23 alters its scaffold function, which could lead to reduced ERK activation in response to signaling. In agreement with this hypothesis, MDA-MB-435 breast cancer cells that over-express NM23 showed reduced ERK activation levels compared with vector alone control transfectants, while a histidine-kinase-deficient mutant of NM23 showed high levels of activated ERK, compared to those of the controlled transfectants (23). Therefore, altered levels of NM23 in metastatic versus non-metastatic tumor cells might impact ERK activation through a complex interaction with the KSR scaffold protein.

4.2. KAI1

The KAI1 gene was isolated originally by microcell mediated chromosome transfer technique (MMCT) as a prostate-specific tumor metastasis suppressor gene. It is located in the p11.2 region of human chromosome 11 (24, 25). When the KAI1 gene was transferred into a highly metastatic prostatic cancer cell line, KAI1-expressing cancer cells were suppressed in their metastatic ability, whereas their primary tumor growth was not affected (24, 25). Therefore, this gene behaves as a classical tumor metastasis suppressor. DNA sequencing analysis of the KAI1 gene revealed that it is identical to CD82, a surface glycoprotein of leukocytes, which encodes 267 amino acids (27). The protein has four hydrophobic and presumably transmembrane domains and one large extracellular N-glycosylated domain. Consistent with the view that KAI1

Table 2. Tumor metastases suppressor genes

Gene	Suppressed in cancer	Location	Function	*In vitro* Motility	*In vitro* Invasion	Tested in Animal	Immunohistochemistry (% negative in met patients)	Reference
Drg-1	Breast, Prostate Colon	22q12.2	Inhibit invasion	↓	↓	+	60% (P=0.04) (Breast), 74% (P=0.003) (Prostate)	102, 105, 106, 108
KAI1	Breast, Prostate	11p11.2	Integrin Interaction, EGFR desensitization	↓	↓	+	94.9% (P=0.025) (Breast), 100% (Prostate)	26, 29
BRMS1	Breast, Melanoma	11q13-q13.2	Gap junctional commiuncation	↓	↓	+		49, 50
KiSS-1	Breast, Melanoma	1q32-q41	G-protein-coupled receptor ligand	↓	↓	+	56% (P=0.482) (Melanoma)	43, 155
NM23	Breast, Prostate Melanoma, Colon	17q21.3	Histidine Kinase	↓	↓	+	66.7% (P=0.013) (Breast), 73% (P=0.289) (Prostate)	17, 156-158
RhoGDI2	Bladder	12p12.3	Regulates Rho & Rac function	↓	↓	+		89
CRSP3	Melanoma	6q22.33-q24.1	Transcriptional coactivator	↓	↓	+		64
MKK4	Prostate, Ovary	17p11.2	MAPKK, JNK kinases	↓	↓	+	67.7% (P<0.0001) (Ovary)	39, 42
VDUP1	Melanoma	1q21.1	Thioredoxin inhibitor					64
E-Cadherin	Breast, Prostate Gastric, Colorectal, Thyroid, Ovary	16q22.1	Inhibit shedding from primary tumor		↑↓	+	47.7% (P=0.147) (Breast), 27.3% (P=0.004) (Prostate)	55, 159, 160
RKIP	Breast, Prostate, Melanoma	12q24.23	Inhibits Raf-mediated MEK phosphorylation	↓	↓	+	39.2% (p=0.367) (Breast)	66, 161
SSeCKS	Prostate	6q24-25.2	Scaffolding protein for PKC & PKA	↓		+		72
Claudin 7	Breast, Cervical, Gastric	17p13	Tight junction protein					76
RRM1	Lung	11p15.5	Ribonucleotide reductase	↓	↓	+		80, 82

is a metastasis suppressor gene, the immunohistochemical analysis of human tumor samples revealed that the expression of the gene in most cases was downregulated during the tumor progression of not only prostate, but also lung (28), breast (29), bladder (30), and pancreatic cancers (31). The down-regulation of the KAI1 gene expression is also correlated with poor survival in patients with those cancers. Furthermore, in a study of prostate tumors including 120 cases, PCR-single-strand conformational polymorphism and microsatellite analyses revealed that the KAI1 expression was down-regulated consistently during the progression of human prostatic cancer and that this down-regulation did not commonly involve either mutation or allelic loss of the KAI1 gene (26). Therefore, the expression of this gene appears to be down-regulated in advanced tumor cells at or post-transcriptional level, presumably by the loss of an activator or gain of a suppressor.

In order to understand the basic regulatory mechanism of the KAI1 gene expression, the 5' upstream region of the KAI1 gene was cloned by screening a human placental genomic library in our laboratory (32). The KAI1 promoter revealed a p53 consensus binding site and in addition, reverse transcription-PCR analysis revealed that the expression of endogenous KAI1 mRNA was augmented significantly by p53. The results of the promoter analysis using a reporter plasmid containing the 5' upstream sequence indicated that the KAI1 gene was indeed positively controlled by p53 at the transcriptional level in prostatic tumor cells. By subsequent analysis of the promoter sequence of the KAI1 gene by site specific mutagenesis and gel-shift mobility assay, we found that the region of 272 bp, which was approximately 860 bp upstream of the transcriptional initiation site, was responsible for this p53 activation (32). Results from these experiments clearly indicate that p53 activates the KAI1 gene at the transcriptional level through its binding to the specific site of the 5' upstream region.

In the search for a specific agent which re-activates the expression of the KAI1 gene, it was found in our laboratory that etoposide, a topoisomerase II inhibitor, is able to activate the expression of the KAI1 gene in a dose-dependent manner in human prostate cancer cell lines as well as in human lung carcinoma cells (33). Our results suggest that the augmentation of the KAI1 gene expression by etoposide is independently controlled by both p53 and c-Jun at the transcriptional level in the human prostate tumor cell lines. Furthermore, treatment of these cell lines with

etoposide resulted in a significant reduction of cellular invasion (33). Because etoposide has been shown to be effective on advanced prostate cancer when used in combination with other regimens, our results provide a further rationale to use this drug as an anti-metastatic agent.

How the KAI1 gene suppresses the metastasis process remains the most intriguing question. Recently, Odintsova *et al.* found that KAI1 physically associates with the EGF receptor and rapidly desensitizes the EGF-induced signal that could lead to suppression of cell migration (34). However, it is yet unclear whether this mechanism indeed accounts for the metastasis suppression *in vivo*. The crucial clue to understand the biochemical function of the KAI1 gene came from the results of the recent studies on T-cell activation. KAI1/CD82 is barely detectable on resting peripheral T and B lymphocytes, while its expression is highly up-regulated upon activation of these cells (35). This up-regulation is associated with some morphologic change and expression of activation markers such as CD82 and MHC II antigens. Lebel-binay *et al.* described that the co-engagement of KAI1/CD82 and TCR by anti-CD82 mAb and anti-CD3 mAb, respectively, was able to activate T cell and that, when a T-cell is stimulated *in vitro* by anti-KAI1/CD82 mAb, KAI1/CD82 appears to transmit a signal which results in tyrosine phosphorylation, a rapid increase in intracellular Ca^{2+} level and IL-2 production (36). Interestingly, this activation was associated with a change in cellular morphology and inhibition of cell proliferation (37). Therefore, it is tempting to speculate that tumor cells of epithelial origin may also employ a similar signal pathway upon activation of KAI1/CD82, which results in growth arrest of tumor cells. In fact, it was shown that NGF was capable of up-regulating the expression of KAI1 in prostate cancer cell lines, and this activation was associated with remarkable down-regulation of cell proliferation *in vitro* and *in vivo* (38). Although it remains to be tested whether the KAI1 up-regulation is coupled to the inhibition of cell proliferation, this raises an attractive possibility that activation of KAI1 may lead to growth suppression in tumor cells of epithelial origin similar to that in cells of haematopoetic origin under certain conditions. Thus the existing information points to a very diverse mode of activation of KAI1/CD82 as revealed in the *in vitro* experiments.

4.3. MKK4

The MKK4 gene was originally identified as a metastasis suppressor for prostate cancer by combination of MMCT and differential expression approaches (39). Following identification of metastasis suppressor activity of a 70cM region on human chromosome 17 in an *in vivo* animal model (40), Yoshida *et al.* examined the genes located within this region and having a biological function suggesting a potential role in metastasis suppression (39). Putative candidate genes that were not specifically retained or expressed by microcell mediated chromosome 17-tranferred prostate cancer cells and normal prostate tissue were eliminated from further consideration. MKK4/SEK1 was identified as a candidate gene based on its physical location, 17p11.2, within the 70-cM metastasis suppressor region, and the fact that its normal cellular function in the stress-activated signaling pathway suggests that alteration of this gene may have pleiotrophic effects on the cell (39). The same group of investigators also observed that expression of the MKK4 gene in a metastatic prostate cancer cell line significantly reduced the number of macroscopic lung metastases in SCID mice as compared with the lungs from control animals, without affecting the primary tumor growth (39). Detailed histological examination of sections from the lungs of tumor-bearing animals indicated that lungs from control mice had large metastatic foci while the lungs from mice bearing MKK4-positive tumors contained significantly small foci. In addition, cuffs of cells approximately two to three layers thick were observed around blood vessels in several of the sections from the MKK4-positive samples, suggesting that the tumor cells may co-opt existing host vasculature for growth (39).

In order to understand the clinical significance of the MKK4 gene in cancer progression, Kim *et al.* performed immunohistochemical studies on clinical samples of prostate cancer (41). The study revealed high levels of MKK4 expression in the epithelial but not the stromal compartment of normal prostatic tissues with a significant down-regulation of expression in the neoplastic tissues, and a statistically significant inverse relationship between Gleason pattern and MKK4 was observed (41). These results demonstrate that the MKK4 gene is consistently down-regulated during prostate cancer progression and supports the notion that disregulation of the MKK4 signaling cascade plays a crucial role in progression of metastatic disease. Similar results have been reported for ovarian cancer as well (42). To test the possibility that down-regulation of MKK4 protein is the result of allelic loss, Kim *et al.* examined the metastatic prostate cancer lesions for loss of heterozygosity (LOH) within the MKK4 locus and found that the downregulation of MKK4 expression in cancer patients does not frequently involve allelic loss or mutation of this gene (41). Although MKK4 is a central molecule in the cell's stress response pathway, how this gene inhibits the metastasis process is yet to be understood.

4.4. KiSS-1

The KiSS-1 gene was originally identified as a metastatic melanoma suppressor gene by combining the aspects of the strategies of both MMCT and differential display. After the introduction of human chromosome 6 into human metastatic melanoma cell lines C8161 or MelJuSo by MMCT resulted in a significant suppression of metastasis without affecting tumorigenicity or local invasiveness, a subtractive hybridization between the highly metastatic parental C8161 and the chromosome 6-C8161 hybrid cells led to the identification of the KiSS-1 transcript (43). The functional role of KiSS-1 in metastasis suppression was evident when the full-length KiSS-1 transfectants suppressed the lung colonization of tumor cells in spontaneous metastasis assay without affecting the growth of the tumor cells *in vivo* (43). Based on the observation that chromosome 1q is frequently deleted in late-stage human breast carcinomas, Lee *et al.* tested whether the KiSS-1 gene that maps to chromosome 1q32-

q41 could suppress metastasis of the human breast carcinoma cell line MDA-MB-435 (44). They found that the expression of KiSS-1 almost completely abrogated the metastatic potential as compared to control cells but did not suppress tumorigenicity. Therefore, KiSS-1 acts as a metastasis suppressor for breast carcinoma as well. The same investigators also noted that metastasis suppression by KiSS-1correlated with a decreased three-dimensional growth of cells in soft agar but invasion and motility were unaffected. Based on the predicted structure of the KiSS-1 protein, these results imply a mechanism whereby KiSS-1 regulates events downstream of cell-matrix adhesion, perhaps involving cytoskeletal reorganization.

Yan *et al.* have recently found that colon carcinoma cell lines HT-1080 stably transfected with a KiSS-1 expression construct, demonstrated substantially lower MMP-9 enzyme activity and *in vitro* invasiveness (45). The lower MMP-9 enzyme activity reflected reduced steady-state mRNA level that in turn was due to attenuated transcription. Moreover they noted that while activation of ERKs and JNKs by phorbol 12-myristate 13-acetate and tumor necrosis factor alpha, respectively, were able to increase the MMP-9 expression, this MMP-9 activation was not antagonized by KiSS-1 expression, suggesting that MAPK pathways modulating MMP-9 synthesis are not the target of KiSS-1 (45). They further observed that although MMP-9 expression is regulated by AP-1, Sp1 and Ets transcription factors, KiSS-1 did not alter the binding of these factors to the MMP-9 promoter. However, NF-κβ binding to the MMP-9 promoter required for expression of this collagenase was reduced by KiSS-1 expression. Diminished NF-κβ binding reflected less p50/p65 in the nucleus secondary to increased I-κβ levels in the cytosols of the KiSS-1 transfectants (45). Their results suggest that KiSS-1 diminishes MMP-9 expression by effecting reduced NF-κB binding to the promoter. Another important clue for KiSS-1 function came from the study of Ohtaki *et al.* (46), who isolated a 54 amino acid peptide from human placenta that turned out to be encoded by Kiss-1 C-terminus and served as the endogenous ligand for an orphan G-protein-coupled receptor (hOT7T175). Named as 'Metastin', this peptide inhibits chemotaxis and invasion of hOT7T175-transfected CHO cells *in vitro* and attenuates pulmonary metastasis of hOT7T175-transfected B16-BL6 melanomas *in vivo*. These results suggest possible mechanisms of action for KiSS-1 and a potential new therapeutic approach. Interestingly, since then, similar results have been reported by two other groups independently (47, 48).

4.5. BRMS1

Several regions spanning the q-arm of chromosome 11 have been found to be associated with a majority of breast cancer cases, the most common being amplifications and deletions involving regions near band 11q13 (49). In particular, reports of high-frequency deletions involving 11q13-q14 in late-stage, metastatic breast carcinomas were suggestive of the existence of a metastasis suppressor gene in this region (49). This was further corroborated by the finding that introduction of a normal human chromosome 11 into the metastatic MDA-MB-435 human breast carcinoma cells by microcell-mediated transfer significantly suppressed metastasis without affecting tumorigenicity. Then, DD-RT-PCR for highly metastatic (MDA-MB-435) parental cells versus the metastasis-suppressed clones led to the identification of three novel cDNA fragments, one of which was identified as BRMS1 (50). Over-expression of BRMS1 in metastatic breast carcinoma cells suppressed metastasis in both spontaneous and experimental breast cancer metastasis models (50). In addition, the same gene was also found to act as a metastasis suppressor for melanoma (51). Stable transfection of BRMS1 in the human melanoma cell lines MelJuSo and C8161.9 did not alter the tumorigenicity of either cell line, but significantly suppressed metastasis compared to vector-only transfectants (51). However, the expression of this gene has not yet been examined in clinical setting.

Toward analyzing mechanisms underlying suppression of metastasis by BRMS1, Samant *et al.* observed that expression of BRMS1 in tumor cells did not make significant difference in adhesion to extracellular matrix components (laminin, fibronectin, type IV collagen, type I collagen) or invasion and only modestly inhibited the motility of the cells and, in some cases, inhibited the ability of the cells to grow in three-dimension in soft agar (52). The results of their study also ruled out the possibility of BRMS1 upregulating expression of other metastasis suppressors, such as NM23, KAI1, KiSS1 or E-cadherin. Some clue regarding function of BRMS1 came from a study by Saunders *et al.*, who reported that transfection and re-expression of BRMS1 restored the ability of human breast carcinoma cells (MDA-435) to form functional homotypic and heterotypic gap junctions (53). Cx43 and Cx26 (connexins) are the predominant gap junction protein in normal breast epithelial tissue but are often reported to be lost in neoplastic breast tissue. Metastatic MDA-MB-435 cells express Cx32 but not Cx43 or Cx26, and restoring BRMS1 expression in this cell line resulted in re-establishment of gap junction but only partly restored Cx43 expression. Based on these observations Saunders *et al.* suggested that re-expression of the BRMS1 gene restores the Cx expression profile from that of a metastatic cell to that more similar to a normal breast epithelial cell and that the composition of gap junctions contributes to metastatic propensity (53).

4.6. E-cadherin

The transmembrane protein E-cadherin (also known as CDH 1) was originally isolated as human uvomorulin by screeing a cDNA library of the human liver (54). The E-cadherin is a calcium-dependent adhesion molecule and constitutes a main component of the adherence junction in epithelia cells. Calcium ions bind to the extracellular domain of E-cadherin at the adhesion site of cell-cell junction, while the intracellular domain of this molecule interacts with beta-catenin to mediate actin binding. E-cadherin also sequesters the function of beta-catenin by blocking nuclear translocation which results in inhibition of transcription of c-myc and cyclin D1 (55). The expression of E-cadherin is generally reduced in a variety of human cancers at advanced stages. It is believed that tumor cells with a low level of E-cadherin can be readily detached from

adjacent cells, and these cells invade and metastasize to other distant organs. Several groups have indeed reported that decreased expression of E-cadherin was associated with a poor prognosis of cancer patients (56). Most importantly, over-expression or maintenance of E-cadherin in invasive cancer cells has been shown to decrease motility and invasiveness (55). Therefore, E-cadherin is considered to function as a metastasis suppressor. Interestingly, E-cadherin has recently been found to be regulated by Snail and Slug (57) that are zinc-finger transcription factors and involved in the process of cell differentiation and apoptosis (58). In breast carcinomas, Snail and Slug have been recently shown to be involved in tumor progression and invasiveness (57), and it is postulated that these proteins repress the expression of E-cadherin (57).

4.7. VDUP1 (TXNIP) and CRSP3

The VDUP1 (Vitamine D3 upregulated protein 1) gene was first identified by the differential display technique as a gene induced by 1,25dihydroxyvitamin D-3 (59). VDUP1 is able to interact with a reduced form of TRN (60), which results in inactivation of TRN. TRN is an inhibitor for apoptosis signal-regulating kinase 1 (ASK-1) which is known to be a central component of stress-induced apoptosis (61). Therefore, VDUP1 is also considered to participate in this signal pathway through the binding to TRN (62). In fact, the expression of VDUP1 has been shown to arrest cell growth of NIH3T3 cells (63). Consistent with these *in vitro* results, immunohisotchemical analyses for tumor specimens revealed that the expression of VDUP and TRN were inversely correlated in many tumors. Over-expression of VDUP1 in a metastatic cell line followed by injection into mice significantly reduced the incidence of lung metastases, suggesting that VDUP1 functions as a metastasis suppressor, The regulatory mechanism of the VDUP1 gene has not been well understood, however, Goldberg and colleagues recently found that VDUP1 is controlled by a transcription factor, CRSP3, and suggested that CRSP3 may also act as an metastasis suppressor and as an up-stream regulator of VDUP and KiSS-1 in human melanoma (64). CRSP3 is known as a co-factor in Sp1 (Specificity protein 1) mediated transcription, and transfection of an expression plasmid of CRSP3 into melanoma cells significantly increased the expression of KiSS1 and VDUP1 genes. Consistent with the notion that CRSP3 is a metastases suppressor gene, over-expression of the CRSP3 gene in metastatic melanoma cells and transplantation of these cells into mice significantly decreased the rate of lung metastasis. Furthermore, the expression of VDUP1 and CRSP3 genes has been shown to be inversely correlated with the progression of melanoma by using quantitative real-time RT-PCR. Therefore, both VDUP1 and CRSP3 apparently act as metastases suppressors via the KISS1 pathway. However, mechanism of metastases suppression by these genes is not yet clear.

4.8. RKIP

Raf kinase inhibitor protein (RKIP) is a member of the phosphatidylethanolamine binding protein (PEBP) family. RKIP encodes a protein which inhibits the Raf/mitogen-activated protein kinase /extracellular signal-regulated kinase (ERK) pathway. This signaling plays an important role in determining cell fate and choosing between diverse responses such as proliferation, differentiation and survival. Interestingly, RKIP was recently identified as a gene significantly down-regulated in a metastatic cell line (C4-2B) of prostate cancer by microarray analyses (65). This result was further corroborated by immunohistochemical examination of clinical tissue samples from cancer patients. It was found that RKIP was usually expressed in benign tissues while it was significantly down-regulated in tumors, especially in metastatic cells. These results suggest that RKIP is associated with suppression of metastasis. In consistence with these data, over-expression of RKIP in a metastatic cell line derived from prostate cancer has been shown to have no effect on cell proliferation or colony-formation ability in soft agar but significantly lower the invasive potential of these cells. Furthermore, overexpression of RKIP drastically decreased the lung metastases of these cells when transplanted into animals without affecting primary tumor growth (66).

Since RKIP is an inhibitor of Raf which phosphorylates MEK and ERK, Fu *et al.* examined the status of phosphorylation of these target proteins in various prostate cancer cell lines and found that both MEK and ERK had higher basal levels of the phosphorylated forms in metastatic cells than in non-metastatic cell line, without significant changes in the total protein level (66). Conversely, the degree of phosphorylation of these target proteins was lower in metastatic cell with RKIP over-expression than in mock transfected cells. In this context, it should be noted that treatment of a metastatic cell line with a MEK kinase inhibitor significantly reduced the invasiveness of the cells, suggesting that RKIP suppresses tumor invasion through MEK activity (66). Interestingly, RKIP has also been shown to promote apoptosis of cancer cell, and low level of RKIP expression significantly increases resistance to chemotherapeutic-induced apoptosis. Thus RKIP also appears to contribute to response of cancer cells in chemotherapy (67).

4.9. SSeCKS

SSeCKS (*Src*-Suppressed C Kinase Substrate) was originally isolated by using PCR-based subtractive hybridization (68, 69). Over-expression of the SSeCKS gene via a retroviral vector caused a significant reduction in cell proliferation compared to a normal control cell or *src*-transfected cell, suggesting that SSeCKS encodes a regulator of mitogenesis. SSeCKS was also known as an orthologue of human Gravin/AKAP12 (A kinase anchor protein 12) which was previously identified as a cytoplasmic antigen recognized in sera from patients with myasthenia gravis (70) and later found to be the cytoplasmic scaffolding protein for protein kinase A and C (71, 72). Recently, Xia *et al.* showed that both RNA and protein levels of SSeCKS/Gravin were significantly decreased in metastatic prostate cancer cell lines of human and rat origin compared to non-metastatic cell lines (72). They also found that the expression of SSeCKS/Gravin inhibited anchorage-independent growth without affecting the cell proliferation. Furthermore, over-expression of

SSeCKS/Gravin in metastatic cell line followed by injecting it into mice significantly decreased the incidence of lung metastasis. Therefore, SSeCKS/Gravin appears to function as a metastasis suppressor.

4.10. Claudin

Claudins, a family of integral membrane proteins, are the basic molecules involved in tight junction structure and function (73). Tight junctions are responsible for controlling the paracellular permeability, cell adhesion and cell polarity. These functions of tight junctions that are often lost in cancer may play a crucial role in tumor growth and metastasis (74). Claudins as prime constituents for tight junctions have been found to be abnormally regulated in human breast and prostate cancers. Claudin-3 and claudin-4 are typically over-expressed in adenocarcinomas including prostate and breast cancers. On the other hand, recent study with pancreatic cancer suggests that claudin-4 functions as an inhibitor of the invasiveness of cells (75). Interestingly, claudin-7 has been found to be significantly down-regulated in invasive ductal carcinomas (IDC) of the breast and there is an inverse correlation between the expression of claudin-7 and cellular discohesion in breast carcinomas (76). These results suggest that claudin-4 and 7 are putatuve metastasis-suppressors, although the role of claudin-4 in the metastasis process remains to be clarified further.

4.11. RRM1

RRM1 (ribonucleotide reductase M1 polypeptide) encodes the regulatory subunit of ribonucleotide reductase which is known to catalyze the rate limiting step of deoxyribonucleotide formation (77-79). RRM1 is located on chromosome 11p15.5 which is often lost in lung cancer at advanced stages and is also significantly associated with metastatic spread in lung cancer patients (80, 81). A recent study by Bepler and colleagues showed that over-expression of RRM1 induced expression of the known tumor suppressor gene, PTEN, in human and mouse cell lines, and also in animal model (82). These authors found that a lung derived stable cell line over-expressing RRM1 significantly reduced migration and invasive abilities compared with a control cell line. The overexpression of RRM1 also strongly induced the expression of PTEN in these cell lines. Importantly, the expression of RRM1 suppressed spontaneous metastasis to the lung and prolonged survival in animals. Therefore, RRM1 appears to function as a metastasis suppressor through induction of PTEN in lung cancer. In fact, immunohistochemical analyses of clinical samples revealed that the expression of RRM1 was significantly correlated with PTEN and RRM2 (ribonucleotide reductase M2 polypeptide) (83). Furthermore, high expression of RRM1 was found to be predictive of long survival independent of tumor stage, performance status, and weight loss (83, 84).

4.12. RhoGD12

The Rho proteins belong to a guanine nucleotide family and they exist in two different forms as being active when bound to GTP and inactive when bound to GDP. RhoGDIs (GDI: GDP-dissociation inhibitor) are the class of proteins that inhibit the dissociation of GDP and stabilizes the inactive form of Rho proteins. RhoGDI2 is a 200 amino acid protein with a molecular weight of 229 kDa and it was first discovered by Leffers *et al.* (85). It was found to be expressed in human and murine hematopoietic tissues, predominantly in B and T lymphocytes (86) as well as in non-hematopoietic neoplastic cells (87). RhoGDI2 is phosphorylated in response to stimulation of T lymphocytes and myelomonocytes cells, and it is involved in inducing hematopoiesis (88). On the other hand, recent study of Gildea *et al.* (89) has shown that inducible expression of exogenous RhoGDI2 in metastatic cells blocked lung metastasis and significantly suppressed invasiveness and motility of cultured cells but did not affect the *in vitro* growth rate, colony formation or *in vivo* tumorigenicity. The intricacy of mechanism by which RhoGDI2 restricts metastasis is yet to be elucidated, but it is speculated that RhoGDI2 suppresses the metastatic process by impeding the tumor cells from invading and colonizing the lung upon reaching the pulmonary vasculature. RhoGDI2 has also been identified as a potent metastatic suppressor in bladder cancer. Therefore, RhoGDI2 is considered as a general metastases suppressor.

4.13. Drg-1

The Drg-1 gene was originally found to be induced *in vitro* by cellular differentiation and hence named as Differentiation-Related-Gene-1 (90). Since then, three more genes, namely, Drg-2, 3 and 4 have been identified that encode proteins highly related to Drg-1 (91, 92). These genes constitute the NDRG gene family although the members vary in the pattern of tissue-specific expression and possibly in function. Drg-1 is identical to the human RTP, cap43 and rit42, and homologous to the mouse genes TDD5 and Ndr1 and rat Bdm1 (93-98). The protein encoded by the Drg-1 gene has a molecular weight of 43 kDa and possesses three unique 10-amino acid tandem repeats at the C terminal end. Analysis of the amino acid sequence predicted that there were seven or more phosphorylation sites, and Drg-1 indeed has been shown to be phosphorylated by Protein Kinase A *in vitro* (99). Drg-1 mRNA is detected in most of the organs, and the level of expression is particularly high in prostate, ovary, intestine and kidney. It was shown that the expression of this gene was repressed by c-myc and N-myc/Max complex *in vitro* (97). On the other hand, p53 was found to be able to induce expression and nuclear translocation of Drg-1 in response to DNA damaging agents (95). The expression of the gene was also augmented by hypoxia and PTEN, and the combination of Drg-1 and PTEN has indeed been shown to be an indicative marker for outcome in patients with both breast and prostate cancers (100-102). In addition, the Drg-1 gene has been shown to be upregulated by hormones such as androgen (96) and by various chemical agents including homocysteine, mercaptoethanol, tunicamycin (98), lysophosphatidylcholine (103), nickel compounds (94) and synthetic retinoids (104). Therefore, the Drg-1 gene is controlled by multiple factors and responsive to various stimuli.

Table 3. Relationship between Drg-1 and other clinical parameters in prostate cancer

Drg-1 expression				
	All	Positive	Reduced	P value
Gleason grade				
≤ 7	38	26	12	
> 7	24	8	16	0.015 [1]
P53				
Wild type	59	32	27	
Mutant	3	2	1	0.8
Differentiation				
Well	16	14	2	
Moderate	19	14	5	
Poor	27	6	21	<0.001[1]
Nuclear grade				
I	32	22	10	
II / III	30	12	18	0.044 [1]
Metastasis status				
Organ confined	40	28	12	
Lymph node	20	5	15	0.003 [1]
Bone	19	5	14	0.006 [1]

[1] Statistically significant. Ref 62

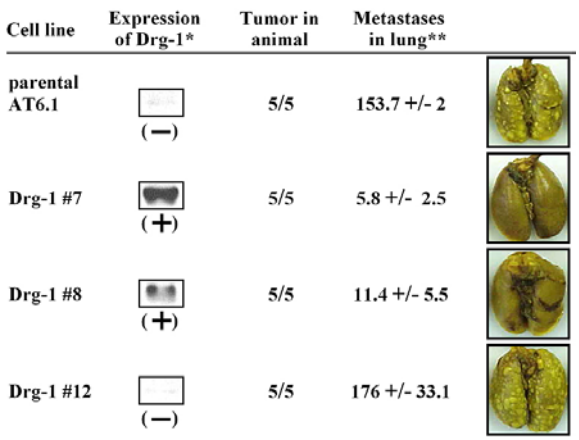

Figure 1. Drg-1 suppresses spontaneous lung metastasis without affecting growth of primary tumor. The parental cell line (AT6.1) and Drg-1-transfected clones (#7, #8, and # 12) were tested for Drg-1 protein expression by Western blot. Each of these cell lines was injected subcutaneously into SCID mice. After 4 weeks, the mice were sacrificed and the lungs were removed. The tumor nodules on the lungs were counted macroscopically. The lungs from mice from each group are shown as examples.

Since the Drg-1 gene is strongly correlated with differentiation and tumor progression is invariably associated with loss of differentiation, we analyzed the Drg-1 expression status in clinical samples of human prostate and breast cancer (105, 106). In both cases, Drg-1 was found to be highly expressed in the epithelial cells of normal glands and ducts where the protein was localized mostly in the cytoplasm. The Drg-1 protein was detected consistently in all cases of normal prostate tissue as well as PIN (Prostatic Intraepithelial Neoplasia) and BPH (Benign Prostatic Hyperplasia), and normal mammary gland cells, while the Drg-1 expression was significantly reduced in the tumor cells of cancer patients (105, 106). In the case of prostate cancer, the reduction in Drg-1 expression correlated significantly with the Gleason grade. A study by Caruso *et al.* also found similar trend of downregulation of Drg-1 expression in prostate cancer, and interestingly, they also observed a significant correlation between Drg-1 expression pattern and ethnic origin of the patients (107). Most interestingly, in both prostate and breast cancers, we observed a significant level of differential expression of Drg-1 between the patients with organ-confined disease and those with metastasis to lymph node or bone (Table 3,106). In case of prostate cancer, the negative correlation of Drg-1 with metastatic spread to lymph node and bone is highly significant, and in fact, is much stronger than the positive correlation with Gleason scores. In breast cancer, a similar and significant negative correlation of Drg-1 with metastases has been observed (106). These results strongly suggest the negative involvement of Drg-1 in the process of invasion and metastasis in both prostate and breast cancer.

The significant inverse correlation of Drg-1 expression with the extent of metastasis at the clinical level raised the next important question as to whether the down-regulation of Drg-1 is cause or result of metastases. To address this issue, we over-expressed the Drg-1 gene in a highly metastatic prostate cell line and implanted it into SCID mice. The result of this experiment indicated that all the clones formed primary tumors in the animals with similar growth rates (data not shown), suggesting that Drg-1 does not have an effect on tumorigenesis and tumor growth. On the other hand, the clones that were positive for Drg-1 expression exhibited a significantly lower incidence of lung metastases compared with the vector-transfected cell line (Figure 1). Similar metastasis suppressor effect of Drg-1 was also observed in colon carcinoma cells by Guan *et al.* (108). Furthermore we observed that Drg-1 significantly suppressed the invasive potential of prostate and breast cancer cells as tested by *in vitro* invasion chamber assay (105, 106). Therefore, evidence from both clinical data and the results of *in vitro* as well as animal experiments overwhelmingly support the notion that Drg-1 is a metastasis suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis. How Drg-1 suppresses the tumor metastases is an intriguing question which is under active investigation.

5. CONCLUSION AND FUTURE DIRECTIONS

The development of metastases is a major obstacle to the successful treatment of a patient with any cancer. Much of the lethality of malignant neoplasms is directly attributable to their ability to develop secondary growths in organs at a distance from the primary tumor mass, while few patients die from their primary neoplasm. Although the clinical importance of tumor metastasis is well recognized, advances in understanding the molecular mechanism involved in metastasis formation have lagged behind other developments in the cancer field. This is because of the fact that metastasis involves multiple steps with high complexity. A possible breakthrough in our understanding of cancer progression has emerged with the hypothesis that tumor metastasis is negatively controlled by tumor metastasis suppressor genes. Thus far fourteen genes have been identified that are defined as tumor metastases suppressors. Almost all of them are also significantly down-regulated in advanced stages in a variety of cancers. However the mechanism of metastases suppression for most of the genes is yet to be clarified. A

cross-talk between these proteins remains an intriguing question. The mechanism of down-regulation of these genes in tumor cells also needs to be addressed. Recent studies in this field have begun to shed light on these questions and understanding the molecular mechanism of tumor metastases suppression would eventually lead to the development of therapeutic approaches to intervene in the process of metastatic disease.

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LETTERS

Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression

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***CD82*, also known as *KAI1*, was recently identified as a prostate cancer metastasis suppressor gene on human chromosome 11p1.2 (ref. 1). The product of *CD82* is KAI1, a 40- to 75-kDa tetraspanin cell-surface protein also known as the leukocyte cell-surface marker CD82 (refs. 1,2). Downregulation of KAI1 has been found to be clinically associated with metastatic progression in a variety of cancers, whereas overexpression of *CD82* specifically suppresses tumor metastasis in various animal models[3]. To define the mechanism of action of KAI1, we used a yeast two-hybrid screen and identified an endothelial cell-surface protein, DARC (also known as gp-Fy), as an interacting partner of KAI1. Our results indicate that the cancer cells expressing KAI1 attach to vascular endothelial cells through direct interaction between KAI1 and DARC, and that this interaction leads to inhibition of tumor cell proliferation and induction of senescence by modulating the expression of TBX2 and p21. Furthermore, the metastasis-suppression activity of KAI1 was significantly compromised in DARC knockout mice, whereas KAI1 completely abrogated pulmonary metastasis in wild-type and heterozygous littermates. These results provide direct evidence that DARC is essential for the function of *CD82* as a suppressor of metastasis.**

We screened the human normal prostate cDNA library using the full-length *CD82* cDNA as bait in a yeast two-hybrid interaction trap[4] and identified Duffy antigen receptor for chemokines (DARC, also known as gp-Fy and encoded by *DARC*) as a potential interactor for KAI1. A liquid β-galactosidase assay quantitatively showed the strength and specificity of the interaction between KAI1 and DARC (**Fig. 1a**). DARC is an approximately 45-kDa, seven-transmembrane protein expressed on vascular endothelium of various organs, as well as on red blood cells and certain epithelial cells[5,6]. It binds chemokines of both C-C and C-X-C families, although ligand binding by DARC does not induce G-protein-coupled signal transduction or Ca^{2+} flux[7,8]. The *DARC* gene has two alleles, Fya and Fyb, which differ only at amino acid residue 44 (ref. 9). Sequence analysis showed that the cloned DNA identified by our screening represents the spliced isoform of the Fyb allele of *DARC* (**Fig. 1b**). To examine the interaction of KAI1 and DARC in mammalian cells, we carried out a coimmunoprecipitation experiment using a highly metastatic prostate carcinoma cell line, AT6.1, which was stably transfected with a Flag epitope–tagged *DARC* gene. The cells were then transiently transfected with a hemagglutinin (HA)-tagged KAI1 plasmid, and the cell lysate was incubated with antibody to Flag. We found that KAI1 coprecipitated with Flag-tagged DARC, suggesting that KAI1 can interact with DARC in mammalian cells (**Fig. 1c**). To localize the regions of KAI1 and DARC that are essential for this interaction, we tested individual domains as well as serial deletions from the amino terminus of KAI1 against full-length DARC target and vice versa in yeast mating assay. Our results indicate that the first intracellular and transmembrane domains of KAI1 are dispensable for this interaction (**Fig. 1d**). On the other hand, deletion of the first extracellular domain of DARC at the amino terminus completely abrogated the interaction, suggesting that the amino terminus of DARC is essential for binding to KAI1 (data not shown).

To assess the relevance of the interaction between KAI1 and DARC, we next examined the localization of DARC in prostate cancer tissue by immunohistochemistry. We found that DARC is highly expressed in the prostate endothelium, particularly in the small veins and venules, as well as in lymphatic vessels, whereas it was undetectable in the epithelial cells and stroma (**Fig. 1e**). The expression of DARC in endothelium was found to be essentially the same in normal, hyperplastic glands and high-grade carcinomas. We observed a similar pattern of expression of DARC in breast and lung cancer samples (data not shown). On the other hand, KAI1 is highly expressed in the normal epithelial cells in these organs, and its expression is substantially reduced in carcinoma, as reported previously[3]. Because expression of *DARC* in these organs is restricted to the vasculature, it is unlikely that KAI1 on epithelial cells interacts with DARC protein in the same cell. Instead, it suggests that such an interaction takes place when cancer cells expressing KAI1 intravasate and encounter the endothelial lining of small blood vessels. Consistent with this hypothesis, a previous study using epifluorescence microscopy detected

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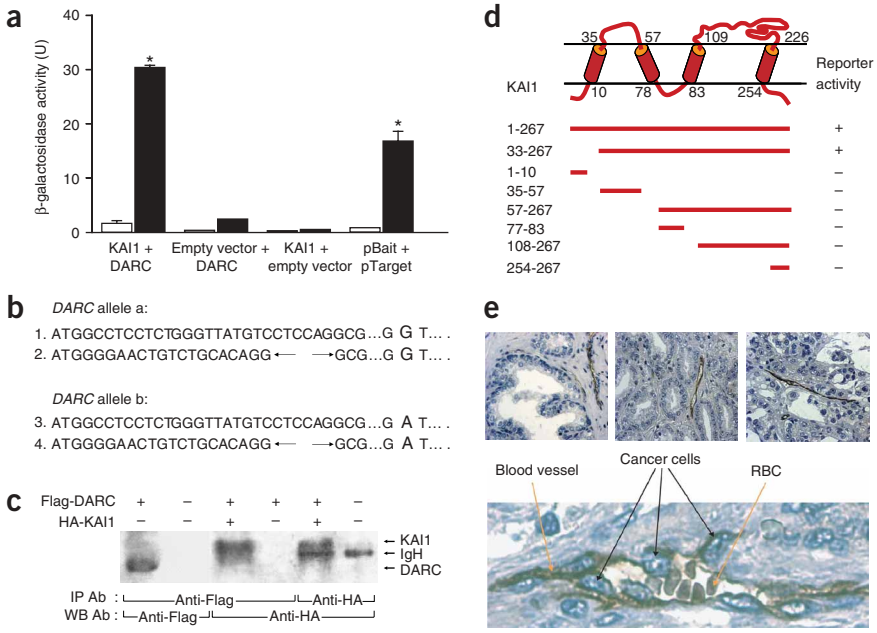

Figure 1 KAI1 interacts with DARC *in vitro*. (**a**) Quantification of interaction between KAI1 and DARC. Yeast cells transformed with an appropriate combination of expression plasmids were grown in minimal medium in the presence of glucose (white bar) or galactose (black bar) as indicated. The β-galactosidase activity is expressed in Miller units (U). pBait and pTarget are a pair of positive control interactors provided by the manufacturer. (**b**) Alleles and splice variants of *DARC*. The junctions of two exons in the biexonic isoforms (#2, #4) are indicated by arrows. (**c**) Coimmunoprecipitation of DARC and KAI1 in mammalian cells. AT6.1/Flag-DARC permanent clone or the parental cell line was tested for DARC expression by immunoprecipitation with monoclonal antibody to Flag covalently crosslinked to agarose beads followed by western blot with monoclonal antibody to Flag (lanes 1, 2). For coimmunoprecipitation, AT6.1/Flag-DARC cells were transiently transfected with HA-tagged KAI1 expression plasmid, proteins were pulled down by Flag-specific agarose beads and KAI1 was detected by western blot with antibody to hemagglutinin (lane 3). To confirm the HA-KAI1 position, the AT6.1/Flag-DARC cells were transfected with HA-KAI1 as above and immunoprecipitation and western bolt were performed with monoclonal antibody to hemagglutinin and protein G agarose followed by western blot with the same monoclonal antibody (lane 5). AT6.1/Flag-DARC cells without KAI1 transfection or parental AT6.1 cells served as negative controls (lanes 4, 6). IgH appeared in lanes 5 and 6, as antibody to hemagglutinin was not crosslinked to the agarose beads during immunoprecipitation. (**d**) Analysis of interactions of various domains of KAI1 with DARC. Regions of KAI1, as indicated by the amino acid sequence numbers, were tested: '+' indicates positive interaction and '–' indicates lack of interaction. (**e**) DARC is expressed only in the vascular endothelium of prostate tissue. Immunohistochemistry was performed on clinical samples using the polyclonal antibody to DARC. Representative fields of normal prostate gland and various grades of prostate carcinoma are shown in the upper panel. DARC is detectable only in the vascular endothelium and red blood cells (RBC). The lower panel represents a magnified view of a blood vessel from a high-grade cancer section.

metastatic tumor cells attached to the endothelium of precapillary arterioles and capillaries in intact mouse lungs[10]. In agreement with this observation, in our archive of specimens, examination of small blood vessels in a high-grade cancer area indicated that cancer cells are often attached to endothelium of blood vessels (**Fig. 1e**).

We next tested the possibility that KAI1 on tumor cells interacts with DARC on endothelial cells by performing a cell-to-cell binding assay *in vitro* in which green fluorescent protein (GFP)-tagged AT6.1 ($KAI1^-$) or AT6.1/Flag-KAI1 ($KAI1^+$) cells were overlaid on $DARC^+$ endothelial cells, human bone marrow endothelial cells (HBMEs) and human umbilical vein endothelial cells (HUVECs). We observed a significantly higher percentage of attachment of $KAI1^+$ cells compared with $KAI1^-$ cells to both types of endothelial cells in a time-dependent manner. Moreover, antibody to KAI1 abrogated this binding, indicating the direct involvement of KAI1 in the process (**Fig. 2a**). We next carried out the same binding assay by overlaying the tumor cells on AT6.1 cells with or without expression of DARC. $KAI1^+$ tumor cells exhibited a binding affinity specifically to the $DARC^+$ AT6.1/Flag-Fy cells (**Fig. 2a**), confirming that the binding of $KAI1^+$ cells to these endothelial cells is indeed due to the expression of DARC. To show a direct interaction between these two membrane proteins in a cell-to-cell manner, we mixed the $KAI1^+$ tumor cells HT-38 and $DARC^+$ HUVECs in the presence of the membrane-impermeable cross-linker 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP), lysed the cells and performed a coimmunoprecipitation experiment. KAI1 coprecipitated with DARC (**Fig. 2b**), whereas another tetraspanin (CD81) did not, indicating a specific interaction between KAI1 and DARC. These results indicate that KAI1-expressing tumor cells can bind to endothelial cells via the interaction between KAI1 and DARC, and suggest the possibility that the metastasis suppressor function of KAI1 is partly due to the trapping of the tumor cells on the endothelial linings of vessels.

It was previously reported that treatment of Jurkat cells with a monoclonal antibody to KAI1 inhibited proliferation of the cells *in vitro*[11]. Therefore, we sought to determine whether this antibody would elicit a similar response in tumor cells expressing KAI1. We found that this antibody significantly inhibited DNA synthesis in $KAI1^+$ prostate tumor cells (**Fig. 2c**). We also obtained similar results for the breast and lung carcinoma cell lines MDA-MB-435 and A549, respectively (data not shown). These results suggest that the growth of KAI1-expressing tumor cells is suppressed when KAI1 on the tumor cell surface is engaged by an appropriate ligand. Consistent with this idea, it was previously reported that exposure of prostate tumor cells to nerve growth factor led to upregulation of KAI1, which was also associated with downregulation of cell proliferation *in vitro*[12]. To examine whether the signaling pathway leading to growth arrest of tumor cells is also activated when KAI1 binds to DARC, we measured the rate of DNA synthesis in tumor cells when they were allowed to contact cells that either did or did not express DARC. The rate of DNA synthesis was significantly reduced only when the cells expressing KAI1 (AT6.1/Flag-KAI1) contacted the $DARC^+$ endothelial cells (HBMEs or HUVECs) or the prostate carcinoma cell line (AT6.1/Flag-DARC; **Fig. 2d**). We obtained similar results for the breast and lung carcinoma cell lines MDA-MB-435 and A549, respectively (data not shown).

To further corroborate the notion of growth arrest of tumor cells upon interaction with DARC on the endothelial cell surface, we mixed GFP-tagged AT6.1 and AT6.1/Flag-KAI1 cells with HBMEs or HUVECs and then selected for GFP^+ tumor cells. We found that the ability of tumor cells to form colonies significantly decreased when

AT6.1/Flag-KAI1 cells (KAI1$^+$), compared with AT6.1 cells (KAI1$^-$), interacted with HBMEs or HUVECs (**Fig. 2e**). We confirmed that this effect is mediated by DARC in the endothelial cells by performing similar experiments in which AT6.1/Flag-KAI1 or AT6.1 cells were mixed with cells with or without DARC expression (AT6.1/Flag-DARC or AT6.1; **Fig. 2e**). Therefore, our data suggest that the interaction between KAI1 and DARC leads to a growth-suppressive effect on the KAI1-bearing cell; thus, the status of KAI1 expression on tumor cells has a key role in determining their fate once they intravasate into the blood vessels.

To examine whether the interaction between KAI1 and DARC is essential for the metastasis suppressor function of KAI1 *in vivo*, we used *Darc*$^{-/-}$ mice[13]. We chose the syngenic metastatic tumor cell lines B16BL6 and B16F10 to establish tumors in these mice and generated several KAI1$^+$ clones or empty-vector transfectants in these cells (**Fig. 3a**). We then injected the B16BL6 derivatives subcutaneously into *Darc*$^{-/-}$ mice and heterozygous and wild-type littermates. We found that primary tumors developed in all mice. The growth rate and final volume of tumors did not significantly vary with the KAI1 level in the tumor cells or with DARC status of the mice (**Table 1**). The KAI1$^+$ clones, however, developed significant numbers of pulmonary metastases in *Darc*$^{-/-}$ mice, whereas metastasis was almost completely abrogated when the same clones were injected in the heterozygous and wild-type littermates (**Fig. 3b** and **Table 1**). The tumor cells lacking KAI1 (B16BL6/vector), however, metastasized equally in all three groups of mice. Thus, in the absence of DARC, even the tumor cells expressing large amounts of KAI1 recapitulated the metastatic phenotype of downregulation of *CD82*. To further corroborate the effect of DARC on the metastatic ability of KAI1-bearing cells, we used an experimental metastasis model in which the metastatic cell line B16F10 stably transfected with KAI1 expression plasmid or an empty vector was injected intravenously into *Darc*$^{-/-}$ mice and their control littermates. The KAI1$^+$ clones resulted in a significantly higher number of pulmonary metastases in the DARC knockout mice, whereas the empty vector transfectant metastasized regardless of the DARC status of the host (**Table 1**). These results support our hypothesis that DARC has a crucial role in the metastasis suppressor function of KAI1 *in vivo*.

DARC is known to be a promiscuous chemokine receptor; however, our *in vitro* data indicate that this function of DARC is not likely to

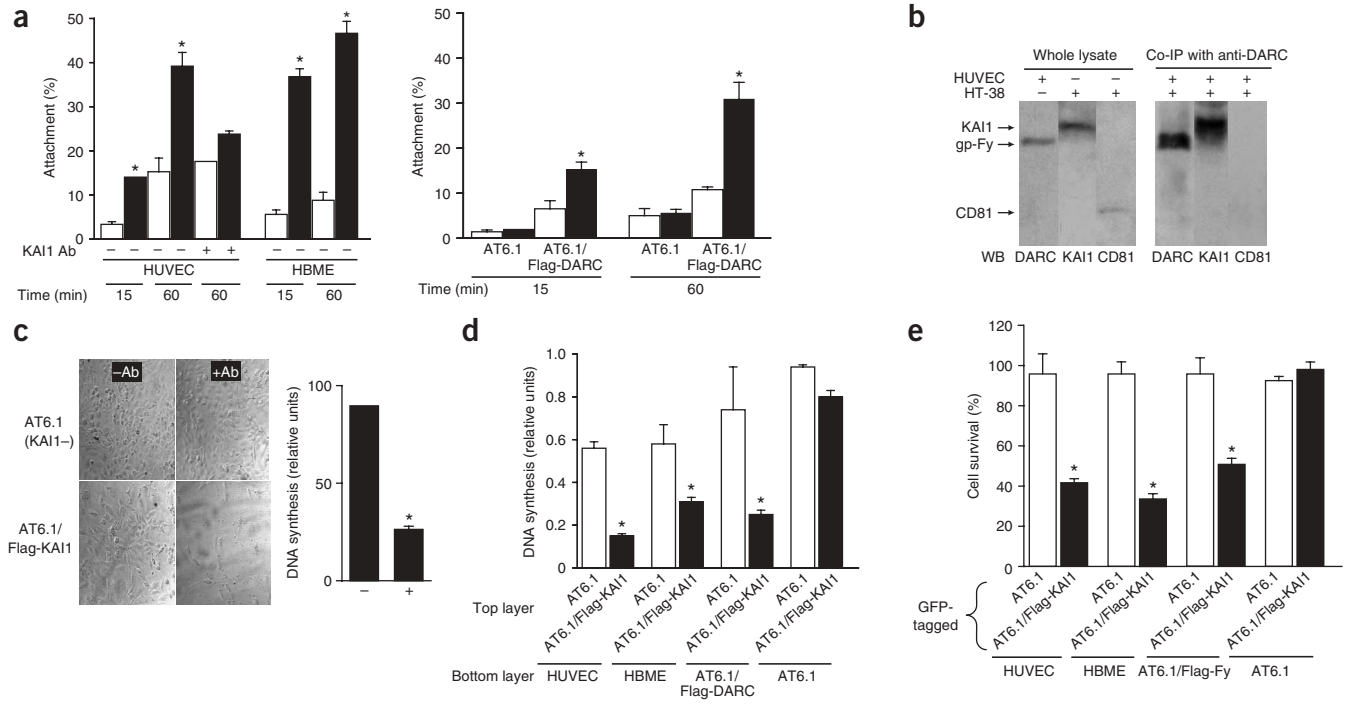

Figure 2 Interaction of KAI1 and DARC leads to growth arrest of cancer cells. (**a**) KAI1 selectively binds to cells expressing DARC in a cell-to-cell binding assay *in vitro*. HBMEs and HUVECs (DARC$^+$; left), AT6.1 and AT6.1/Flag-DARC (right) were grown to confluency. Then, approximately 10^3 cells of AT6.1 tagged with GFP (KAI1$^-$, white bar) or AT6.1/Flag-KAI1 tagged with GFP (KAI1$^+$, black bar) were added on the confluent cell layers, in the presence (+) or absence (–) of monoclonal antibody to KAI1 as indicated. After 15 min or 1 h, wells were washed and the percentage of attachment was calculated as described in Methods. (**b**) Endogenous KAI1 and DARC coimmunoprecipitate in mammalian cells. Lanes 1–3: expression level of KAI1 and CD81 in HT-38 cells and level of DARC in HUVECs were tested by western blot using antibodies to KAI1, CD81 and DARC, respectively. Lanes 4–6: HT-38 and HUVECs were mixed in the presence of a cell-impermeable crosslinker DTSSP for 30 min followed by immunoprecipitation with DARC antibody and western blot with antibodies to KAI1, DARC or CD81, as indicated. (**c**) Monoclonal antibody to KAI1 inhibits growth of KAI1$^+$ prostate epithelial cells. AT6.1 (KAI1$^-$) or AT6.1/Flag-KAI1 (KAI1$^+$) were seeded and monoclonal antibody to KAI1 was added to the wells indicated by '+ Ab' and the rate of DNA synthesis was measured. (**d**) Suppression of DNA synthesis by DARC in prostate cancer cells. DARC$^+$ endothelial cells (HUVECs, HBMEs) and cells with or without DARC expression (AT6.1, AT6.1/Flag-DARC; bottom layer) were grown to full confluency and incubated with 30 μM mitomycin C for 18 h. The cells were then washed extensively, and AT6.1 (white bars) or AT6.1/Flag-KAI1 (black bar) cells (top layer) were added on the monolayer, ^{3}H-thymidine was added to the wells and the incorporation of radioisotopes into DNA of the attached cells was assayed. (**e**) Growth arrest in prostate cancer cells caused by interaction between KAI1 and DARC. Prostate cancer cells expressing both *CD82* and *GFP* genes (AT6.1/Flag-KAI1, black bars) or cells expressing only GFP (AT6.1, white bars) were mixed with cells with or without DARC expression for 1 h followed by plating in the presence of hygromycin, which allowed growth of only GFP-tagged AT6.1 or AT6.1/Flag-KAI1 cells. After 5 d, the number of colonies was counted under a fluorescent microscope. *$P < 0.05$.

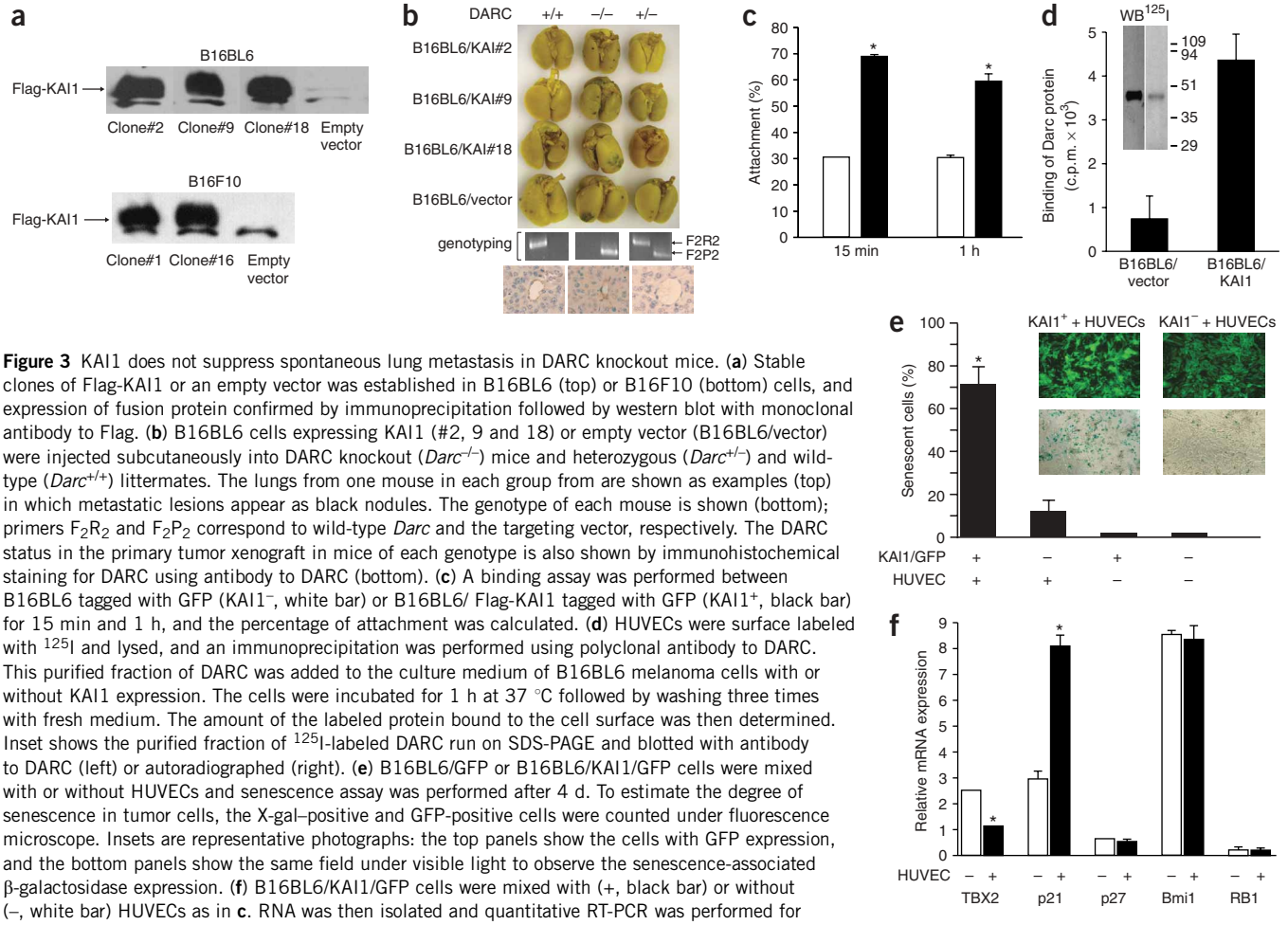

Figure 3 KAI1 does not suppress spontaneous lung metastasis in DARC knockout mice. (**a**) Stable clones of Flag-KAI1 or an empty vector was established in B16BL6 (top) or B16F10 (bottom) cells, and expression of fusion protein confirmed by immunoprecipitation followed by western blot with monoclonal antibody to Flag. (**b**) B16BL6 cells expressing KAI1 (#2, 9 and 18) or empty vector (B16BL6/vector) were injected subcutaneously into DARC knockout (*Darc*$^{-/-}$) mice and heterozygous (*Darc*$^{+/-}$) and wild-type (*Darc*$^{+/+}$) littermates. The lungs from one mouse in each group from are shown as examples (top) in which metastatic lesions appear as black nodules. The genotype of each mouse is shown (bottom); primers F_2R_2 and F_2P_2 correspond to wild-type *Darc* and the targeting vector, respectively. The DARC status in the primary tumor xenograft in mice of each genotype is also shown by immunohistochemical staining for DARC using antibody to DARC (bottom). (**c**) A binding assay was performed between B16BL6 tagged with GFP (KAI1$^-$, white bar) or B16BL6/ Flag-KAI1 tagged with GFP (KAI1$^+$, black bar) for 15 min and 1 h, and the percentage of attachment was calculated. (**d**) HUVECs were surface labeled with ^{125}I and lysed, and an immunoprecipitation was performed using polyclonal antibody to DARC. This purified fraction of DARC was added to the culture medium of B16BL6 melanoma cells with or without KAI1 expression. The cells were incubated for 1 h at 37 °C followed by washing three times with fresh medium. The amount of the labeled protein bound to the cell surface was then determined. Inset shows the purified fraction of ^{125}I-labeled DARC run on SDS-PAGE and blotted with antibody to DARC (left) or autoradiographed (right). (**e**) B16BL6/GFP or B16BL6/KAI1/GFP cells were mixed with or without HUVECs and senescence assay was performed after 4 d. To estimate the degree of senescence in tumor cells, the X-gal–positive and GFP-positive cells were counted under fluorescence microscope. Insets are representative photographs: the top panels show the cells with GFP expression, and the bottom panels show the same field under visible light to observe the senescence-associated β-galactosidase expression. (**f**) B16BL6/KAI1/GFP cells were mixed with (+, black bar) or without (–, white bar) HUVECs as in **c**. RNA was then isolated and quantitative RT-PCR was performed for various senescence-associated genes including *TBX2*, *CDKN1A*, *CDKN1B*, *BMI1* and *RB1*.

have a role in the metastasis-suppression action of KAI1. Rather, DARC seems to directly engage in the interaction with KAI1, which triggers an unknown signal pathway of growth arrest. To obtain mechanistic insight into the interaction between KAI1 and DARC that led to metastasis suppression in our *in vivo* model system, we first carried out a cell-to-cell binding assay using melanoma cells. We found that B16BL6 cells overexpressing KAI1 exhibited a significantly higher binding to the endothelial cells over different time points (**Fig. 3c**), which is consistent with our observation in the case of prostate tumor cells. We then tested the binding of ^{125}I-labeled purified fraction of DARC to the cell surface of B16BL6 melanoma cells with or without KAI1 expression. A significantly ($P < 0.05$) higher amount of DARC bound to the melanoma cells expressing KAI1 compared with the empty vector transfectant (**Fig. 3d**), supporting our notion that KAI1 and DARC interact at the surface of the tumor cells. Such interaction leads to growth arrest of tumor cells (**Fig. 2d,e**). However, we did not detect apoptosis in the KAI1$^+$ tumor cells by TUNEL assay upon coculturing with DARC$^+$ cells (data not shown). We therefore examined whether the interaction with DARC leads to senescence in the KAI1$^+$ tumor cells by mixing HUVECs with GFP-tagged B16BL6 cells with or without KAI1 expression. We found that a significant percentage of KAI1$^+$ tumor cells underwent senescence as a result of interaction with HUVECs (**Fig. 3e**). Furthermore, we found that expression of the senescence-associated gene *TBX2* was reduced and *CDKN1A* (encoding p21) was upregulated in these cells upon interaction with HUVECs, whereas *CDKN1B* (encoding p27), *BMI1* or *RB1* did not show any appreciable change in expression level (**Fig. 3f**). Notably, several previous publications showed a potential link between tumor progression and senescence[14–18]. Particularly consistent with our results, TBX2 has been found to inhibit senescence by directly repressing p21 expression in melanoma cells, suggesting that the TBX2-p21 pathway has a crucial role in tumor progression[19].

Collectively, our results indicate that when tumor cells dislodge from the primary tumor and intravasate into the blood vessels, tumor cells expressing KAI1 attach to the endothelial cell surface, whereby KAI1 interacts with DARC. This interaction transmits a senescent signal to the tumor cells, whereas those that lost KAI1 expression proliferate in the circulation, potentially giving rise to metastases. Notably, KAI1 as a tetraspanin was previously shown to interact with several other cell-surface proteins including α4β1 integrin[20]. The presence of these integrins on tumor cells promotes attachment to vascular endothelial cells[21]. Therefore, the association of integrin and KAI1 may have a part in the KAI1-DARC interaction, although this possibility needs to be explored further. Nonetheless, our model of the mechanism of action of KAI1 explains how KAI1 suppresses metastasis without affecting formation of primary tumors. It highlights a previously unappreciated function of *DARC* and identifies DARC as a new candidate for potential therapeutic intervention for metastatic cancer.

Table 1 Spontaneous and experimental metastases of B16BL6/KAI1 cells in DARC knockout mice

Spontaneous metastases of B16BL6/KAI1 cells in DARC knockout mice

Clone #	KAI1 expression	Tumor volume (mean ± s.e.m.)			Incidence of pulmonary metastasis			
		Darc$^{+/+}$	*Darc*$^{-/-}$	*Darc*$^{+/-}$	*Darc*$^{+/+}$	*Darc*$^{-/-}$	*Darc*$^{+/-}$	*P* value
2	Positive	4.9 ± 0.03	4.5 ± 0.02	4.5 ± 0.01	2/15 (13.3%)	9/15 (60%)	1/15 (6.7%)	0.02[a], 0.008[b]
9	Positive	4.6 ± 0.05	4.5 ± 0.03	4.9 ± 0.04	1/15 (6.7%)	6/13 (46.2%)	1/15 (6.7%)	0.05[a], 0.05[b]
18	Positive	4.5 ± 0.05	4.2 ± 0.03	3.9 ± 0.04	0/13 (0%)	6/12 (50%)	0/13 (0%)	0.04[a], 0.04[b]
Empty vector	Negative	4.9 ± 0.05	4.8 ± 0.05	4.9 ± 0.03	6/15 (40%)	5/14 (35.7%)	5/14 (35.7%)	0.8[a], 0.89[b]

Experimental metastases of B16F10/KAI1 cells in DARC knockout mice

Clone #	KAI1 expression	Number of pulmonary metastases			*P* value
		Darc$^{+/+}$	*Darc*$^{-/-}$	*Darc*$^{+/-}$	
1	Positive	4.7 ± 2.4 ($n = 9$)	47.86 ± 5.9 ($n = 7$)	2.8 ± 0.8 ($n = 6$)	<0.001[a], <0.001[b]
16	Positive	4.4 ± 2.4 ($n = 7$)	32.14 ± 3.6 ($n = 7$)	9.4 ± 2.7 ($n = 5$)	<0.001[a], 0.001[b]
Empty vector	Negative	40.0 ± 8.4 ($n = 5$)	56.0 ± 11.8 ($n = 5$)	32.5 ± 4.8 ($n = 6$)	0.3[a], 0.08[b]

[a]Comparison between *Darc*$^{-/-}$ and *Darc*$^{+/+}$. [b]Comparison between *Darc*$^{-/-}$ and *Darc*$^{+/-}$.

METHODS

Yeast two-hybrid screening. We cloned full-length *KAI1* cDNA cloned into the yeast vector pEG202-NLS (Origene Technologies) as bait, and performed yeast two-hybrid screening and mating assay according to the manufacturer's protocol.

Quantitative β-galactosidase assay. We performed the β-galactosidase assay (Miller test) as previously described[22].

Cell culture. The rat prostatic carcinoma cell line AT6.1, the human breast carcinoma cell line MDA-MB-435, HBMEs and the mouse melanoma cell lines B16BL6 and B16F10 were provided by C. Rinker-Schaeffer (University of Chicago), B.E. Weissman (University of North Carolina at Chapel Hill), K. Pienta (University of Michigan Medical School) and I.J. Fidler (M.D. Anderson Cancer Center), respectively. We purchased the human lung epithelial carcinoma cell line A549 and colon carcinoma cell line HT-38 from American Type Tissue Culture Collection. We cultured the cells in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS, 250 nM dexamethasone and antibiotics. We obtained HUVECs from Clonetics and cultured them in endothelial growth medium (EGM, Clonetics) as per the manufacturer's instruction.

Immunoprecipitation and western blot. For coimmunoprecipitation experiments using the AT6.1 cells, approximately 48 h after transfection, we harvested cells and lysed them in ice-cold lysis buffer (1% NP40, 10 mM Tris, pH 8.0, 150 mM NaCl, 3 mM $MgCl_2$, 2 mM PMSF) for 45 min and centrifuged them at maximum speed for 15 min. For immunoprecipitation with monoclonal antibody to Flag, we used Flag-specific M2 affinity gel (Sigma). For immunoprecipitation with antibody to hemagglutinin, we incubated the lysate with monoclonal antibody to hemagglutinin (Boehringer Mannheim) and used protein G-Sepharose beads. After immunoprecipitation, we thoroughly washed the beads, and analyzed bound proteins by western blot using monoclonal antibody to hemagglutinin or monoclonal antibody to Flag (Sigma) at dilutions of 1:400 and 1:500, respectively. For coimmunoprecipitation of endogenous KAI1 and DARC, we mixed the KAI1$^+$ tumor cell line HT-38 with DARC$^+$ HUVECs in the presence of the cell-impermeable cross-linker DTSSP for 30 min at 24 °C. We lysed the cells in the same lysis buffer as above, centrifuged them and immunoprecipitated the lysate with rabbit polyclonal antibody to DARC in the presence of protein G agarose beads. After immunoprecipitation, we analyzed bound proteins by western blot using antibody to DARC (1:500), mouse monoclonal antibody to KAI1 (1:1,000, a gift from O. Yoshie, Shionogi Institute for Medical Science) or mouse monoclonal antibody to CD81 (1:20, Chemicon).

Immunohistochemistry. We carried out immunohistochemical analysis on paraffin-embedded, surgically resected specimens of prostate, breast and lung, using polyclonal antibody to DARC. Briefly, we deparaffinized sections, rehydrated them and heated them at 80 °C for 20 min in 25 mM sodium citrate buffer (pH 9) for antigen exposure. We treated sections with 3% H_2O_2 to block endogenous peroxidase activity and then incubated them with primary antibody (1:50 dilution) for 1 h at 24 °C. After washing in Tris-buffered saline/0.1% Tween-20, we incubated sections with horseradish peroxidase–conjugated rabbit-specific IgG (Dako Corp.). We washed sections extensively, and applied DAB substrate chromogen solution followed by counterstaining with hematoxylin. The Southern Illinois University Institutional Review Board approved obtaining human specimens for this study.

Cell-to-cell binding assay. We seeded HBMEs, HUVECs, AT6.1 or AT6.1/Flag-DARC (DARC$^+$ permanent clone established in AT6.1) cells in 24-well plates and grew them to full confluency. We trypsinized cells used for overlaying (AT6.1/GFP and AT6.1/Flag-KAI1/GFP, or B16BL6/GFP and B16BL6/Flag-KAI1/GFP) and resuspended them in RPMI medium, and added 10^3 cells on the confluent bottom cell layers in the presence or absence of antibody to KAI1. After 15 min or 1 h, we washed the wells with RPMI medium three times and incubated the cells for 12 h at 37 °C. The numbers of cells attached on confluent monolayers were then counted by observing GFP signal under a confocal microscope and the percentage of attached cells was calculated. For each data point, experiments were performed in triplicate wells and ten random fields were counted in each well.

Treatment of tumor cells with monoclonal antibody to KAI1. We seeded approximately 10^3 cells of AT6.1 and AT6.1/Flag-KAI1 in 96-well plates. We then added ^{3}H-thymidine with or without monoclonal antibody to KAI1 (provided by H. Conjeaud, Cochin Hospital) to the wells, which we then incubated at 37 °C for 48 h. The ^{3}H-thymidine incorporation by the AT6.1/KAI1 cells was normalized with respect to the incorporation by the AT6.1 cells. Each experiment was performed in triplicate.

Measurement of DNA synthesis. We cultured HUVECs, HBMEs, AT6.1 and AT6.1/Flag-DARC cells to confluency and then treated them with mitomycin C for 18 h to block DNA synthesis. After washing the wells extensively with RPMI media, we seeded 10^3 AT6.1 cells that did or did not express KAI1 (AT6.1/Flag-KAI1 or AT6.1) on the monolayer of mitomycin C–treated cells and added ^{3}H-thymidine to the wells. We incubated the cells at 37 °C for 48 h, then washed the wells with RPMI media three times and measured the incorporation of ^{3}H-thymidine in the attached cells. The rate of DNA synthesis by the cells

seeded on monolayers was normalized by that of cells seeded directly on the plastic plate. Each experiment was performed in triplicate.

Colony formation assay. We trypsinized HUVECs, HBMEs, AT6.1 and AT6.1/DARC cells, resuspended them in RPMI medium and mixed them with AT6.1 cells, which expressed the gene encoding GFP with or without KAI1 (AT6.1 or AT6.1/Flag-KAI1, both GFP tagged), for 1 h, then plated the mixture in RPMI medium containing hygromycin. The GFP-tagged AT6.1 or AT6.1/Flag-KAI1 cells were also plated without mixing with HUVECs, HBMEs, AT6.1 or AT6.1/DARC cells for the purpose of normalization. We incubated the cells at 37 °C for 5 d and counted the number of colonies expressing GFP under the fluorescence microscope. The number of colonies formed by GFP-tagged AT6.1 or AT6.1/Flag-KAI1 mixed with HUVECs, HBMEs, AT6.1 and AT6.1/DARC cells was normalized with the number of colonies formed by the GFP-tagged cells alone. Each experiment was done in triplicate.

***In vivo* metastasis assay.** For spontaneous metastasis assay, we injected approximately 0.5×10^6 cells/0.2 ml of PBS of various B16BL6 clones subcutaneously in the dorsal flank of the DARC knockout mice as well as heterozygous and wild-type littermates. We monitored mice daily for the growth of primary tumor. After 6 weeks, mice were killed, tumor volume was calculated using the equation Volume = (Width + Length)/2 × width × length × 0.5236, and metastatic lesions were counted macroscopically. For experimental metastasis assay, we injected approximately 0.5×10^6 cells/0.2 ml PBS of various B16F10 clones intravenously into the tail vein of the DARC knockout mice as well as control littermates. Mice were killed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically. All protocols were approved by the Southern Illinois University Institutional Review Board.

***In vitro* binding assay.** The DARC$^+$ cells were surface labeled with ^{125}I using Iodo-beads (Pierce) according to the manufacturer's protocol. We lysed the cells and immunoprecipitated them using antibody to DARC and protein G agarose. We washed the agarose beads extensively to remove unbound proteins and eluted the bound proteins using 0.1 M glycine, pH 3.5, immediately followed by neutralization with 0.5 M Tris, HCl, pH 7.4, 1.5 M NaCl. This eluate was further concentrated by Centricon P10. B16BL6 cells with or without KAI1 expression were seeded in 24-well plates and grown to confluency. We added the purified protein to the cells in culture and 48 h later, washed the wells three times with fresh medium and determined the amount of the bound protein.

Senescence assay. We trypsinized B16BL6/GFP or B16BL6/Flag-KAI1/GFP cells, resuspended them in medium and mixed them with the DARC$^+$ HUVECs for 1 h followed by plating the mixture. We also plated the GFP-tagged cells without mixing with HUVECs as control. We incubated the cells at 37 °C for 4 d. We then performed a senescence assay using a senescence-associated β-galactosidase detection kit (Calbiochem) according to the manufacturer's instruction, and counted the X-gal–positive and GFP-positive cells under a fluorescence microscope.

Real-time RT-PCR. We mixed B16BL6/Flag-KAI1/GFP cells with or without the DARC$^+$ HUVECs for 1 h, and then plated the mixture and incubated it at 37 °C for 4 d. We isolated total RNA from the cells and reverse-transcribed it. We then amplified the cDNA with a pair of mouse-specific forward and reverse primers for the following genes: *TBX2* (forward, 5′-CACCTTCCGCACCTATGTC-3′; reverse, 5′-CAAACGGAGAGTGGGCAGCGTT-3′), *CDKN1A* (forward, 5′-CCGTGGACAGTGAGCAGTT-3′; reverse, 5′-CCAATCTGCGCTTGGAGTGA-3′), *BMI1* (forward, 5′-AATCCCCACTTAATGTGTGTC-3′; reverse, 5′-TCACCTCTTCCTTAGGCTTCTC-3′), *CDKN1B* (forward, 5′-GTGGACCAAATGCCTGACT-3′; reverse, 5′-GGCGTCTGCTCCACAGTG-3′), *RB1* (forward, 5′-TGATGAAGAGGCAAACGTGG-3′; reverse, 5′-TGGCCCACAGCGTTAGCAAAC-3′) and β-actin. We performed PCR using DNA engine opticon2 system (MJ Research) and the Dynamo SYBR Green qPCR Kit (Finnzyme Corp). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 15 min followed by 30 cycles of PCR using the following profile: 94 °C for 30 s; 57 °C for 30 s; 72 °C for 30 s.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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The Tumor Metastasis Suppressor Gene *Drg-1* Down-regulates the Expression of Activating Transcription Factor 3 in Prostate Cancer

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Abstract

The tumor metastasis suppressor gene *Drg-1* has been shown to suppress metastasis without affecting tumorigenicity in immunodeficient mouse models of prostate and colon cancer. Expression of Drg-1 has also been found to have a significant inverse correlation with metastasis or invasiveness in various types of human cancer. However, how Drg-1 exerts its metastasis suppressor function remains unknown. In the present study, to elucidate the mechanism of action of the *Drg-1* gene, we did a microarray analysis and found that induction of Drg-1 significantly inhibited the expression of activating transcription factor (ATF) 3, a member of the ATF/cyclic AMP–responsive element binding protein family of transcription factors. We also showed that Drg-1 attenuated the endogenous level of ATF3 mRNA and protein in prostate cancer cells, whereas Drg-1 small interfering RNA up-regulated the ATF3 expression. Furthermore, Drg-1 suppressed the promoter activity of the *ATF3* gene, indicating that Drg-1 regulates ATF3 expression at the transcriptional level. Our immunohistochemical analysis on prostate cancer specimens revealed that nuclear expression of ATF3 was inversely correlated to Drg-1 expression and positively correlated to metastases. Consistently, we have found that ATF3 overexpression promoted invasiveness of prostate tumor cells *in vitro*, whereas Drg-1 suppressed the invasive ability of these cells. More importantly, overexpression of ATF3 in prostate cancer cells significantly enhanced spontaneous lung metastasis of these cells without affecting primary tumorigenicity in a severe combined immunodeficient mouse model. Taken together, our results strongly suggest that Drg-1 suppresses metastasis of prostate tumor cells, at least in part, by inhibiting the invasive ability of the cells via down-regulation of the expression of the *ATF3* gene. (Cancer Res 2006; 66(24): 11983-90)

Introduction

Drg-1 (differentiation-related gene-1), also known as *Ndrg1* (N-*myc* down-regulated gene 1), was originally identified as being strongly up-regulated on induction of differentiation in colon carcinoma cell lines (1). This gene has been shown recently to play an important role in the context of human cancer progression. We have shown that Drg-1 suppresses lung metastasis of prostate cancer cells without affecting the growth of primary tumor in a severe combined immunodeficient (SCID) mouse model, strongly indicating the role of the *Drg-1* gene as a metastasis suppressor for prostate cancer (2). Drg-1 has also been shown to exert a similar metastasis-suppressive effect in colon cancer cells in a mouse model (3). Consistent with our *in vivo* results, we and others have found that expression of the *Drg-1* gene is inversely correlated with Gleason grades in prostate cancer, and importantly, this down-regulation is more significant in patients with metastasis to lymph nodes than those with organ-confined disease (2, 4). Notably, we have observed similar inverse correlation of Drg-1 expression with metastasis in breast carcinoma patients (5). More recently, Drg-1 expression has been found to have a significant inverse correlation with depth of invasion in pancreatic adenocarcinoma patients as well (6). These data indicate that *Drg-1* indeed is a critical player in the process of tumor metastasis and it is imperative to understand the mechanism of action of this gene.

The *Drg-1* gene encodes a 43-kDa cytoplasmic protein that has several noticeable features, although the biochemical function of the protein is yet largely unknown. Amino acid sequence of the Drg-1 protein reveals three serine phosphorylation sites, five calmodulin kinase 2 phosphorylation sites, five myristoylation sites, three protein kinase C phosphorylation sites, one tyrosine phosphorylation site, one thioesterase site, and one phosphopantotheine attachment site. It has been shown that protein kinase A and calmodulin kinase 2 are indeed involved in the phosphorylation of this protein *in vitro* (7, 8). At the COOH-terminal end of the Drg-1 protein, there are three tandem repeats of the amino acids G-T-R-S-R-S-F-T-H-T-S. Murray et al. showed recently that the COOH-terminal stretch of the Drg-1 protein serves as a substrate for phosphorylation by serum- and glucocorticoid-induced kinase 1, which then primes it for phosphorylation by glycogen synthase kinase 3 (9, 10). However, the physiologic relevance of such phosphorylation remains largely unknown. In addition, based on potentiometric and spectroscopic studies, Zoroddu et al. (11) have proposed that this COOH-terminal stretch may be important for nickel binding. The amino acid sequence of Drg-1 also indicates the presence of a prominent β-hydrolase fold, although it may not be enzymatically functional (12). Thus, the Drg-1 protein presents several interesting features; however, the biochemical function of this protein in the context of tumor metastasis suppression remains to be elucidated.

As an initial step toward understanding how *Drg-1* suppresses the process of tumor metastasis, we have done a microarray

Note: S. Bandyopadhyay and Y. Wang contributed equally to this work.

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analysis to find the downstream target of this gene. Here, we present evidence that Drg-1 suppresses expression of the *activating transcription factor* (*ATF*) *3* gene in prostate and breast tumor cells and that this regulation occurs largely at the transcriptional level. We also show that Drg-1 and ATF3 expression inversely correlate at the clinical level and that ATF3 promotes invasion of prostate tumor cells *in vitro* and spontaneous metastasis *in vivo*.

Materials and Methods

Cell lines. Human prostate cancer cell line PC3 was obtained from American Type Culture Collection (Manassas, VA). Human prostate cancer cell lines, ALVA and PC3MM, were kindly provided by Drs. W. Rosner (Columbia University, New York, NY) and I.J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX), respectively. Rat prostate cancer cell line AT2.1 was a gift from Dr. C. W. Rinker-Schaeffer (University of Chicago, Chicago, IL). All cell lines were cultured in RPMI 1640 supplemented with 10% FCS, streptomycin (100 μg/mL), penicillin (100 units/mL), and dexamethasone (250 nmol/L) at 37°C in a 5% CO_2 atmosphere.

Expression plasmids and transfection. Drg-1 cDNA was a generous gift from Dr. S.W. Lee (Beth Israel Deaconess Medical Center, Boston, MA). To create the mammalian constitutive expression plasmid pcDNA3/Drg-1, the cDNA was PCR amplified where the forward primer included the Kozak sequence and *Eco*R1 linker and the reverse primer included a *Xho*I linker. The PCR product was cloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) using standard techniques. The expression of Drg-1 in the transfected cells was confirmed by Western blot. To construct an inducible expression vector of *Drg-1*, the cDNA of this gene was cloned into the pCMV-Tag2 expression vector (Stratagene, La Jolla, CA), and the in-frame fusion between the Flag tag and Drg-1 and the expression of the fused protein were confirmed by sequencing as well as Western blot. The Flag-Drg-1 cDNA was then PCR amplified and cloned into the *Eco*RV/*Xho*1 site of the inducible expression vector pcDNA5/TO (Invitrogen) using standard techniques. To create a cell line with inducible Drg-1 expression, the tetracycline-inducible system T-Rex (Invitrogen) was used. First, the human prostate cancer cell line PC3MM was transfected with the regulatory plasmid pcDNA6/TR encoding the Tet repressor, and a stable cell line (PC3MM/Tet) was generated by blasticidin selection (2 μg/mL). Then, the pcDNA5/TO/Flag-Drg-1 expression plasmid was stably transfected into the PC3MM/Tet cell line and permanent clones were generated by blasticidin and hygromycin selection, and the resultant clones were designated as PC3MM/Tet-Flag-Drg-1. The induction of Drg-1 by tetracycline in this system was confirmed by Western blot. To create a mammalian expression plasmid of ATF3 (pcDNA3/ATF3), the ATF3 cDNA was excised from the pCG-ATF3 expression plasmid (13) and subcloned into the *Eco*R1/*Hin*dIII site of the mammalian expression vector pcDNA3 using standard techniques. Construction of the pATF3-CAT reporter plasmid containing the −1850 to +34 region of the ATF3 gene was described before (14). For DNA transfection into ALVA, PC3MM, MDA-435, and MCF7 cells, LipofectAMINE 2000 (Invitrogen) was used, whereas PC3 cells were transfected by TransIT-TKO transfection reagent (Mirus Corp., Madison, WI).

Microarray analysis. The PC3MM/Tet-Flag-Drg-1 cells were treated with 1 μg/mL tetracycline or an equal volume of 70% alcohol when the cells reached 80% confluency. Forty-eight hours after induction, the cells were collected and total RNA was prepared using RNeasy mini kit (Qiagen, Valencia, CA). The RNA was converted to cDNA and biotinylated followed by hybridization to an Affymetrix (Santa Clara, CA) Human Gene Array at the W.M. Keck Foundation Biotechnology Research Laboratory at Yale University.

Real-time reverse transcription-PCR. Forty-eight hours after transfection of appropriate plasmid DNA or forty-eight hours after induction by tetracycline, total RNA was isolated from the cells and reverse transcribed using random hexamer and MuLV reverse transcriptase (Applied Biosystems, Foster, CA). The cDNA was then amplified with a pair of forward and reverse primers for the *ATF3* gene (5′-AGTCACTGTCAGCGACAGAC and 5′-TGCTCTCGTTCTTGAG) and for the human *β-actin* gene. PCRs were done using DNA Engine Opticon2 System (MJ Research, Waltham, MA) and the Dynamo SYBR Green qPCR kit New England Biolabs (Ipswich, MA). The thermal cycling conditions composed of an initial denaturation step at 95°C for 5 minutes followed by 30 cycles of PCR using the following profile: 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds.

Western blot. Forty-eight hours after transfection, the cells were collected and subjected to Western blot using antibodies against Drg-1 (1:5,000), ATF3 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), or tubulin (1;1,000; Upstate Biotechnology, Lake Placid, NY). The membranes were incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies and visualized by Enhanced Chemiluminescence Plus system (Amersham Life Sciences, Piscataway, NJ).

Small interfering RNA transfection. Four individual small interfering RNAs (siRNA) against the *Drg-1* gene were synthesized by Dharmacon (Chicago, IL) and combined into one pool (SMARTpool). One siRNA duplex targeting the *green florescence protein* (*GFP*) gene was used as a negative control in all the experiments. The siRNA was transfected into the tumor cell lines using the TransIT-TKO transfection reagent according to the manufacturer's protocol.

Chloramphenicol acetyl transferase reporter assay. Forty-eight hours after transfection of plasmid DNAs, the cells were collected and then subjected to chloramphenicol acetyl transferase (CAT) assay as described previously (14). The reaction was done and acetylated [^{14}C]chloramphenicol was quantified with a PhosphorImager (Packard Instruments, Meriden, CT).

***In vitro* motility and invasion assay.** For motility assay, 10^5 cells were added to the cell culture inserts (24-well format) with microporous membrane without any extracellular matrix coating (Beckton Dickinson, Bedford, MA). Seven hundred microliter of RPMI 1640 containing 20% fetal bovine serum were added to the bottom chamber. They were then incubated for 24 hours at 37°C, and the upper chamber was removed. The cells that invaded through the membrane were stained with tetrazolium dye and counted under microscope. For *in vitro* invasion assay, the working method was similar as described above, except that the cell culture inserts to which the cells were seeded were coated with Matrigel (Beckton Dickinson). Triplicate tests were done in each case.

Tumor specimens and immunohistochemical staining. Formaldehyde-fixed and paraffin embedded tissue specimens from 64 prostate cancer patients were obtained from surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan). Four-micron-thick sections were cut from the paraffin blocks of prostate tumors and mounted on charged glass slides. The sections were deparaffinized and rehydrated, and antigen retrieval was done by heating the slide in 25 mmol/L sodium citrate buffer (pH 9.0) at 80°C for 30 minutes (for Drg-1) or by autoclaving the slide in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 minutes (for ATF3). The slides were incubated overnight at 4°C with anti-Drg-1 rabbit polyclonal antibody (1:200) or anti-ATF3 rabbit polyclonal antibody (1:50; Santa Cruz Biotechnology). The sections were incubated with the HRP-conjugated anti-rabbit secondary antibody, and 3,3′-diaminobenzidine substrate chromogen solution (Envision Plus kit, DAKO Corp., Carpinteria, CA) was applied followed by counterstaining with hematoxylin. Results of the immunohistochemistry for Drg-1 and ATF3 were judged based on the intensity of staining combined with percentage of cells with positive staining, and the grading of the Drg-1 and ATF3 expression was done by two independent persons (S.B. and K.W.).

Spontaneous metastasis assay. To examine the growth rate and metastatic ability of the prostate tumor cells expressing ATF3 in animals, 0.5×10^6 cells in 0.2 mL of PBS were injected s.c. in the dorsal flank of 5-week-old SCID mice (Harlan Sprague-Dawley, Indianapolis, IN). Mice were monitored daily, and the tumor volume was measured as an index of the growth rate. Tumor volume was calculated using the equation, volume = (width + length) / 2 × width × length × 0.5236. The doubling time of tumor during the fastest growing period was calculated by measuring the tumor volume every 4 days. Mice were sacrificed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically.

Statistical analysis. For *in vitro* experiments and animal studies, one-way ANOVA was used to calculate the *P* values. The association between Drg-1 and ATF3 expression was calculated by χ^2 analysis. For all of the

statistical tests, the significance was defined as $P < 0.05$. SPSS software was used in all cases.

Results

Drg-1 attenuates the expression of the *ATF3* gene *in vitro*. To identify the downstream target of the Drg-1 pathway, we did a microarray analysis using the Affymetrix human gene array U133A. For this purpose, we first established tetracycline-inducible expression of Drg-1 in the prostate cancer cell line PC3MM (PC3MM/Tet-Flag-Drg-1), and expression of the *Drg-1* gene was induced by treating the cells with tetracycline or solvent alone for 48 hours. The RNA was then extracted from these cells, converted into cDNA, and hybridized to the microarray. The results of our microarray analyses indicated that the *ATF3* gene, a member of ATF/cyclic AMP–responsive element binding protein (CREB) transcription factor family, was most significantly suppressed by induction of the *Drg-1* gene. Because recent evidence suggests potential involvement of the *ATF3* gene in tumor progression (15–20) and we are particularly interested in the genes up-regulated by suppression of Drg-1 because these may serve as potential therapeutic targets, we decided to examine further the roles of ATF3 in the metastasis suppressor function of Drg-1. First, to confirm the results of the microarray analysis, we induced Drg-1 expression in the same cell line (PC3MM/Tet-Flag-Drg-1) and examined the level of ATF3 mRNA and protein by real-time reverse transcription-PCR (RT-PCR) analysis and Western blot, respectively. As shown in Fig. 1*A*, Drg-1 significantly abrogated ATF3 expression at both mRNA and protein levels, suggesting that induction of Drg-1 indeed leads to attenuation of expression of the *ATF3* gene.

To examine the effect of Drg-1 on endogenous ATF3 expression in various prostate tumor cells, the Drg-1 expression plasmid (pcDNA3/Drg-1) or the empty pcDNA3 vector was transiently transfected into the PC3MM and ALVA cells and the level of ATF3 protein was examined by Western blot. As shown in Fig. 1*B*, Drg-1 attenuated the ATF3 expression in a dose-dependent manner in these cell lines, whereas the empty vector did not have any notable effect. We observed similar effect of Drg-1 on ATF3 expression in breast cancer cell lines MCF-7 and MDA-435 (data not shown). In a complementary approach, we introduced Drg-1 siRNA or GFP siRNA in the prostate cancer cells, PC3MM and ALVA, and as

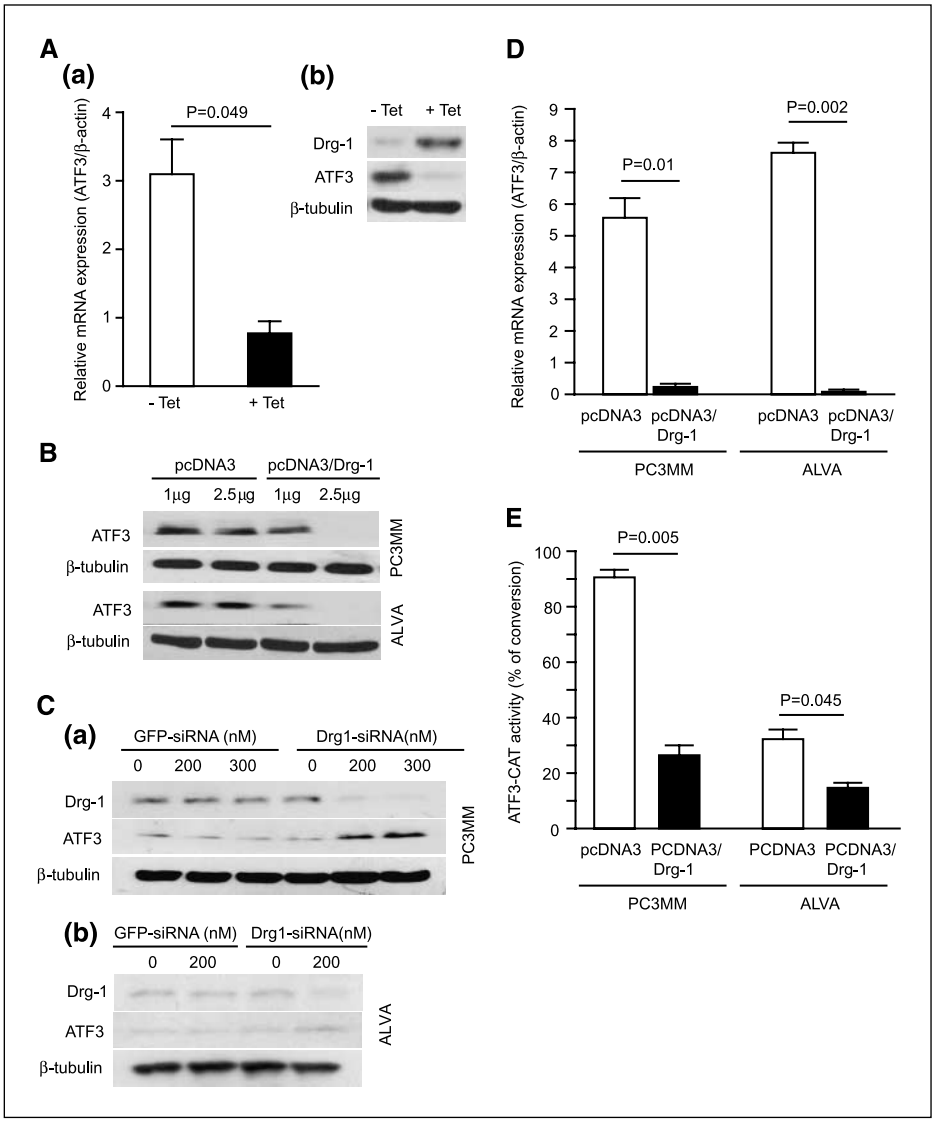


Figure 1. Drg-1 down-regulates ATF3 expression. *A,* PC3MM cells with tetracycline-inducible Drg-1 expression system were cultured with (+*Tet*) or without (−*Tet*) tetracycline. The cells were harvested, and RNA was prepared and subjected to quantitative RT-PCR (*a*). Another set of cells from identical experiment was lysed and expression of Drg-1, ATF3, and tubulin was examined by Western blot analyses (*b*). *B,* empty vector pcDNA3 or Drg-1 expression vector, pcDNA3/Drg-1, at the indicated amounts, was transfected into the prostate cancer cell lines PC3MM and ALVA. Forty-eight hours after transfection, cells were lysed and Western blot was done using antibodies against ATF3 and tubulin. *C,* siRNA for Drg-1 or GFP was synthesized as described in Materials and Methods. Various amounts of the siRNA, as indicated, were transfected into PC3MM (*a*) and ALVA (*b*) cells. After 72 hours, cells were lysed and the lysates were examined by Western blot with antibodies for Drg-1, ATF3, and tubulin. *D,* the prostate tumor cells, PC3MM and ALVA, were transfected with 2.5 μg empty vector pcDNA3 or Drg-1 expression vector, pcDNA3/Drg-1. Forty-eight hours after transfection, total RNA was prepared from these cells and the expression of the *ATF3* and *β-actin* genes was examined by real-time quantitative RT-PCR. *E,* a CAT-reporter plasmid (ATF3-CAT) containing the ATF3 promoter region (−1850 to +34) was cotransfected with Drg-1 expression plasmid (pcDNA3/Drg-1) or empty vector (pcDNA3) into PC3MM and ALVA prostate cancer cells. Forty-eight hours later, the cells were harvested and lysed and the lysates were then assayed for the CAT activity. Acetylated chloramphenicol was resolved on thin-layer chromatography plate and each spot was quantified. A reporter plasmid containing the β-actin promoter (β-actin-CAT) was used as a control.

shown in Fig. 1*C*, the Drg-1 siRNA specifically abrogated expression of the *Drg-1* gene, which led to concomitant up-regulation of the ATF3 expression in these cells. These data strongly suggest that Drg-1 plays a crucial role in regulation of the *ATF3* gene, and down-regulation of Drg-1 in tumor cells results in augmentation of ATF3 expression. To determine whether the down-regulation of ATF3 by Drg-1 is mediated at the RNA level, pcDNA3/Drg-1 or pcDNA3 empty vector was transiently transfected into the above prostate cancer cell lines, and the level of ATF3 mRNA was measured by a real-time quantitative RT-PCR. Consistent with the results of our microarray analysis, we found that Drg-1 significantly attenuated ATF3 expression in these cells, indicating that Drg-1 down-regulates the *ATF3* gene at the mRNA level (Fig. 1*D*). We observed similar trends in MCF-7 and MDA-435 breast cancer cells as well (data not shown). To further examine whether down-regulation of ATF3 expression by Drg-1 is mediated at the transcriptional level, prostate cancer cell lines, PC3MM and ALVA, were cotransfected with Drg-1 expression vector (pcDNA3/Drg-1) or an empty vector (pcDNA3) and ATF3-CAT reporter plasmid, and the CAT reporter assay was done. As shown in Fig. 1*E*, we found that the ATF3-CAT reporter activity was significantly attenuated by Drg-1, thereby strongly suggesting that Drg-1 negatively controls the expression of the *ATF3* gene at the transcriptional level.

ATF3 augments invasiveness of prostate cancer cells *in vitro*. Because we have found previously that stable overexpression of Drg-1 suppresses the invasiveness of several prostate tumor cells *in vitro* (2), we sought the possibility that ATF3 may be involved in motility and invasive properties of cells. We therefore transiently transfected ATF3 into human prostate cancer cell lines, PC3MM and ALVA, and assayed for the motility and invasiveness of the cells. As shown in Fig. 2*A* and *B*, expression of ATF3 significantly augmented invasive ability of these cells when they were tested by an *in vitro* Matrigel assay, whereas the motile ability of the cells remained virtually identical to the cells transfected with empty vector. These data indicate that ATF3 promotes the invasive ability of prostate cancer cells *in vitro* and suggest that attenuation of ATF3 expression by Drg-1 suppresses the invasiveness of tumor cells. To further corroborate this idea, the above prostate cancer cells were transiently transfected with Drg-1 expression vector (pcDNA3/Drg-1), and the invasiveness of these cells was tested. As shown in Fig. 2*C*, Drg-1 strongly inhibited the invasive ability of these cells compared with the empty vector transfectants. Taken together, these results strongly suggest that Drg-1 suppresses the invasive ability of cells via inhibition of expression of the *ATF3* gene.

Expression of Drg-1 and ATF3 correlates in clinical setting. The result of our *in vitro* experiments prompted us to examine whether there is any correlation between Drg-1 and ATF3 expression levels in the clinical setting. Toward that end, we did an immunohistochemical analysis on an archive of 64 prostate cancer tissue samples. The results of the immunohistochemistry

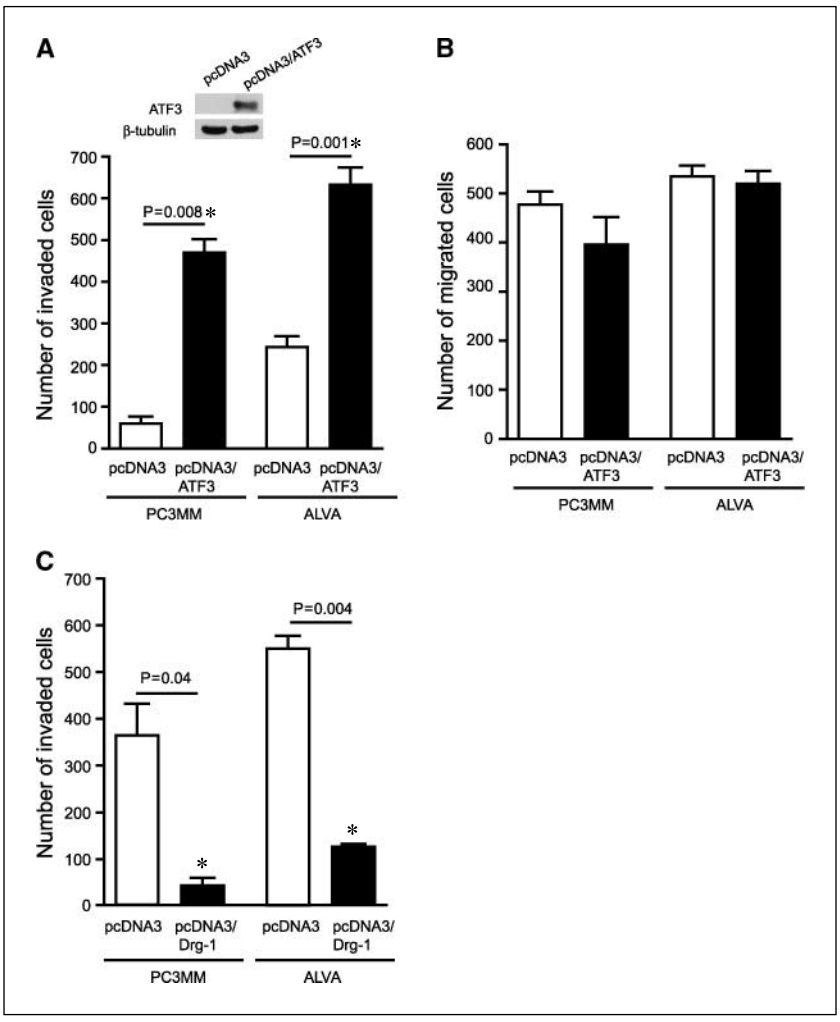
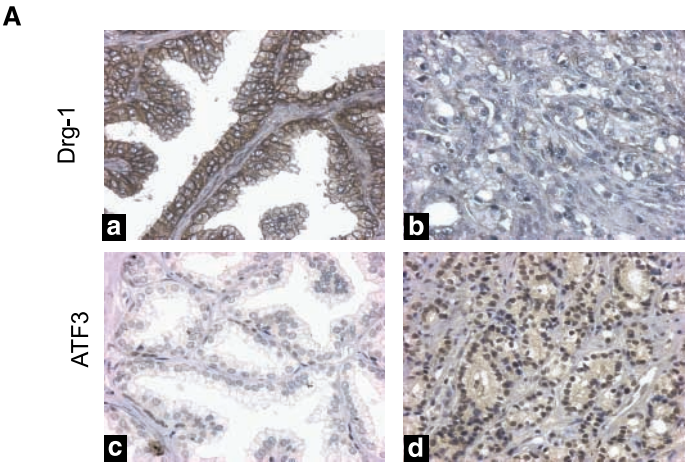


Figure 2. The effect of ATF3 and Drg-1 on the invasiveness and motility of prostate cancer cells *in vitro*. ATF3 expression plasmid (pcDNA3/ATF3) or an empty vector (pcDNA3) was transfected into PC3MM and ALVA prostate cancer cells, and 48 hours after transfection, these cells were examined for invasiveness (*A*) using Matrigel-coated invasion chamber and for motility (*B*) using cell culture inserts without any reconstituted extracellular matrix. Expression of ATF3 protein following transient transfection of the expression construct (*A, inset*). *C,* PC3MM and ALVA cells were transfected with the empty vector pcDNA3 or the Drg-1 expression plasmid (pcDNA3/Drg-1). Forty-eight hours after transfection, the cells were subjected to invasion chamber assay as described in (*A*) above. All assays were done in triplicate. *, $P < 0.05$, statistically significant difference.



B

Relationship between ATF3 and Drg-1 expression

		ATF3 nuclear expression		P value
	All (64)	positive (40)	negative (24)	
Drg-1 expression				
Positive	38	19 (50%)	19 (50%)	
Reduced	26	21 (80.8%)	5 (19.2%)	0.025*
Metastasis status				
Negative	39	19 (48.7%)	20 (51.3%)	
Positive	25	21 (84%)	4 (16%)	0.010*

		ATF3 cytoplasmic expression		P value
	All (64)	positive (27)	negative (37)	
Drg-1 expression				
Positive	38	14 (%)	24 (%)	
Reduced	26	13 (%)	13 (%)	0.430
Metastasis status				
Negative	39	16 (41%)	23 (59%)	
Positive	25	11 (44%)	14 (56%)	1.000

Figure 3. Immunohistochemical analysis of Drg-1 with respect to ATF3 in human prostate cancer. *A,* immunohistochemistry for Drg-1 and ATF3 was done on paraffin tissue sections from prostate cancer patients. Drg-1 immunostaining in a representative field from a prostate cancer patient sample showing normal prostatic gland (*a*) and poorly differentiated prostate carcinoma cells (*b*). A consecutive section from the same tissue specimen is shown after immunostaining for ATF3 (*c* and *d*). Note the strong nuclear expression of ATF3 in carcinoma (*d*). *B,* nuclear expression of ATF3 inversely correlates with Drg-1 expression and positively associates with metastasis status. Immunohistochemistry was done on prostate tissue specimens as described in Materials and Methods. *, $P < 0.05$, statistically significant correlation, as tested by χ^2 analysis.

revealed that Drg-1 is expressed strongly in the cytoplasm of the epithelial cells of normal ducts and glands in prostate tissue sections, whereas the poorly differentiated tumor cells in the same specimen had significantly reduced level of Drg-1 (Fig. 3*A, a* and *b*). Notably, Drg-1 expression was undetectable in the nuclei of normal or cancerous tissue or in the stromal cells. On the other hand, in the epithelial cells of normal ducts and glands, the ATF3 protein weakly expressed mostly in the cytoplasm, whereas, in cancerous cells, there was a notable increase and shift of the ATF3 expression in the nuclei (Fig. 3*A, c* and *d*). Statistical analysis indicated that there was no correlation between Drg-1 and cytoplasmic ATF3 expression; however, Drg-1 and nuclear ATF3 had a significant inverse correlation ($P = 0.025$; Fig. 3*B*). Of 26 patients who had reduced Drg-1 expression, 21 (80.8%) patients also exhibited strong nuclear expression of ATF3, whereas only 5 (19.2%) patients were negative for ATF3 nuclear expression. More importantly, among 25 cases that were positive for bone metastases, 21 (84%) also had positive expression of nuclear ATF3, indicating that ATF3 expression had a significant positive correlation with distant metastasis ($P = 0.010$). The results of this immunohistochemical analysis are therefore consistent with our notion that Drg-1 down-regulates the expression of ATF3 and suggest a possibility that Drg-1 suppresses metastases of prostate cancer cells by inhibiting the expression of the *ATF3* gene.

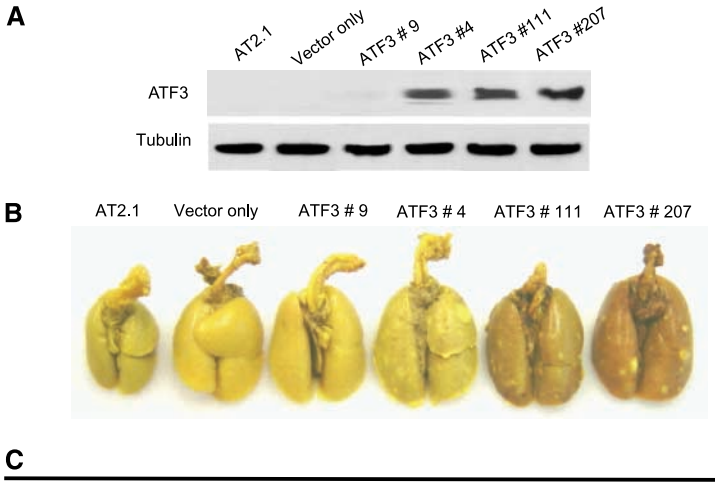
ATF3 promotes spontaneous lung metastasis of prostate cancer cells *in vivo*. To investigate the role of ATF3 in primary tumor growth as well as metastasis *in vivo*, the Dunning rat prostate cancer cell line AT2.1, AT2.1 stably overexpressing ATF3, or AT2.1 transfected with the vector alone was individually injected s.c. into the dorsal flanks of SCID mice. As shown in Fig. 4*A*, Western blot analysis indicated that the clones 4, 111, and 207 expressed ATF3 protein, whereas AT2.1 parental cells, the vector-transfected clone, and the clone 9 did not have any detectable level of ATF3 expression and therefore served as negative controls. The mice were monitored for the formation and the growth rate of tumors for a period of 4 weeks after the inoculation of the cells, and they were sacrificed at the experimental period. Their lungs were then removed and the number of metastatic lesions was grossly counted (Fig. 4*B*). As shown in Fig. 4*C*, all the clones and the parental cells formed primary tumors in the animals with similar

growth rates during the 4-week period, indicating that ATF3 did not have an effect on tumorigenesis and growth of prostate cancer cells. AT2.1 has a poor metastatic propensity and consistently, AT2.1, the vector transfectant cell line, or the clone lacking ATF3 expression (ATF3 clone 9) produced a few metastatic nodules in the lungs. The clones that had stable expression of ATF3 (ATF3 clones 4, 111, and 207), however, significantly augmented the degree of lung metastases causing an average of ~40 metastatic foci in the lungs. These results strongly suggest that ATF3 has the ability to promote the metastatic process of prostate cancer cells without affecting primary tumorigenicity *in vivo*.

Discussion

Metastasis is the ultimate cause of death in any type of cancer, and yet this aspect of the cancer biology remains poorly understood because of the complexity of the metastatic process. Metastasis is negatively controlled by the tumor metastasis suppressor genes that by definition suppress the metastatic dissemination of cancer cells without affecting tumorigenicity. Till date, only a few genes have been identified that clearly meet these criteria (i.e., *NM23, KAI1, Kiss1, Brms1, MKK4, RhoGD12, RKIP, CRSP3, SSeCK, TXNIP/VDUP-1, Claudin-4*, and *RRM1*; refs. 21–24). Recent work by our group and others has indicated that Drg-1 serves as one of such metastasis suppressor genes, although mechanistic insight into how Drg-1 suppresses metastasis is still lacking (2, 3, 5). In this report, we have shown that Drg-1 blocks the metastasis process by attenuating the expression of the *ATF3* gene at mRNA and protein levels and that this regulation occurs for the most part at the transcriptional level.

ATF3 belongs to the mammalian ATF/CREB family of transcription factors (13). Members of this family of proteins bind to a consensus DNA sequence (TGACGTCA) and possess the basic region/leucine zipper (bZip) domain (13). ATF3 acts as a transcriptional repressor as a homodimer, although the same protein functions as a transcriptional activator in heterodimeric form (25–27). ATF3 has been shown to regulate the expression of several genes, including *Thrombospondin, Decorin, E-selectin*, gluconeogenic enzymes, *Gadd153/Chop10*, and *Osteocalcin* via CREB/activator protein-1 (AP-1) motifs (28–32). *ATF3* is a stress-inducible gene that also affects cell cycle progression and apoptosis in various ways and has been implicated recently in the development of cancer. The *ATF3* gene is localized on human chromosome 1q32 within a region that is found to be frequently amplified in esophageal squamous cell carcinoma (33). ATF3 was also reported recently to be highly expressed in classic Hodgkin's lymphoma but not in the non-Hodgkin's lymphoma, and blockade of ATF3 by siRNA reduced proliferation and viability of the Hodgkin's lymphoma cells (15). A separate study by Iyengar et al. (16) also suggested that ATF3 promotes mammary tumorigenesis by induction of antiapoptotic program. Consistently, antisense ATF3 oligonucleotide was shown to inhibit growth of the colon cancer cell line HT29 *in vivo*, although it had no effect on the growth of these tumor cells *in vitro* (18). These reports strongly suggest a positive role of the *ATF3* gene toward advancement of cancer. It is of interest to note that other members of the *ATF* family have been



C

Cell line	ATF3 [a]	Tumor incidence [b]	*In vivo* doubling time	Lung metastasis (mean+/−S.E.) [c]	P value
AT2.1	−	5/5	4.1+/−0.5	5+/−1.9	
Vector only	−	5/5	3.9+/−0.3	5.2+/−3.3	0.96
ATF#9	−	4/4	3.7+/−0.8	2.5+/−0.9	0.51
ATF#4	+	5/5	4.3+/−0.3	39+/−11.3	0.02*
ATF#111	+	5/5	3.7+/−0.2	32+/−9.0	0.02*
ATF#207	+	5/5	2.5+/−0.2	46+/−3.2	<0.001*

a. ATF3 expression was examined by western blot.
b. Number of tumor-bearing SCID mice / no. of tumor-inoculated SCID mice.
c. Number of metastatic lesions on lungs per SCID mouse.

Figure 4. ATF3 augments spontaneous lung metastasis without affecting growth of primary tumor. *A,* the parental cell line (*AT2.1*), cells transfected with vector (*Vector only*), and ATF3-transfected clones (*#9, #4, #111*, and *#207*) were tested for ATF3 and tubulin protein expression by Western blot using anti-ATF3 rabbit polyclonal antibody and anti-tubulin mouse monoclonal antibody, respectively. Each of these cell lines was injected s.c. into SCID mice (five mice per group). After 4 weeks, the mice were sacrificed and the lungs were removed. The tumor nodules on the lungs were counted macroscopically. *B,* the lungs from two mice from each group are shown as examples. *C,* the number of tumor-bearing mice, primary tumor growth rate, and metastases formation are summarized.

implicated in this process as well. For example, strong nuclear expression of ATF2 is associated with metastasis and poor survival in melanoma patients, and ATF4 has been reported to increase cisplatin resistance of human cancer cell lines (34, 35). However, recent growing body of evidence indicates that much still remains to be learned about the complex roles of the genes of the *ATF* family in the context of tumor progression. In addition to its growth-promoting effect, ATF3 was found to be induced following DNA damage in HCT-116 and RKO colon carcinoma cells and suppressed the growth of HeLa cells (36). In a separate study, ATF3 synergized with curcumin to induce apoptosis in squamous cell carcinoma cell line MDA-1986 (37). Furthermore, Bottone et al. (38) have shown that overexpression of ATF3 in HCT-116 colon carcinoma cells decreased focus formation and invasiveness *in vitro* and also reduced growth of xenograft tumor, although the antisense ATF3 had no effect *in vivo*. Thus, ATF3 plays a complex role in tumor progression, and it is possible that some of the apparent contradictions in terms of the function of the *ATF3* gene arise at least in part due to difference in the cellular context.

In this report, we show that the *ATF3* gene promotes invasion of prostate tumor cells *in vitro*, although migration of these cells was not affected. Previously, Ishiguro et al. showed that antisense ATF3 oligonucleotide inhibited invasion and migration of HT29 colon cancer cells *in vitro*, whereas ATF3 expression correlated with the depth of invasion in clinical samples of colon cancer (18–20). In addition, ATF3 expression was found to be higher in human colon and stomach cancer cell lines that were established from metastatic sites than those derived from primary tumor sites (20). Consistently, the highly metastatic melanoma cells B16F10 has been reported to express ATF3 at a much higher level than its low-metastatic counterpart B16F1 (17). These results are in good agreement with our finding and point toward a proinvasive and prometastatic function of the *ATF3* gene. Furthermore, we and others have shown previously that Drg-1 suppresses invasion and metastasis of colon and prostate cancer cells, and Drg-1 expression has a significant inverse correlation with metastasis in prostate and breast cancer (2, 3, 5). Notably, as shown in this report, we have observed a significant inverse correlation between Drg-1 and ATF3 expression and a positive correlation between ATF3 expression and distant metastases in clinical samples of prostate cancer. These results, together with the results of our *in vitro* experiments, strongly support our notion that the metastasis suppressor gene *Drg-1* attenuates the invasive ability of cells by inhibiting the expression of the *ATF3* gene. How ATF3 promotes invasion remains to be understood at the cellular and molecular levels. Stearns et al. (39) have reported recently that direct binding of ATF3 to the matrix metalloproteinase-2 (MMP-2) promoter leads to interleukin-10-mediated suppression of MMP-2. However, Yan et al. (40) showed previously that ATF3 represses MMP-2 expression by interfering with p53-dependent transactivation of this gene, independent of the CREB/AP-1 binding motif on the MMP-2 promoter. Consistently, they did not find any effect of ATF3 on the MMP-2 expression in cells where p53 level was low. The PC3MM cells (metastatic derivative of PC3) used in our study are p53 null; therefore, ATF3 is considered to affect the invasive ability of these cells in MMP-2-independent manner (41, 42).

We have shown that ATF3 promotes pulmonary metastases of poorly metastatic Dunning rat prostate tumor cells (AT2.1) in a SCID mouse model without affecting the growth of the primary tumor (Fig. 4). This is the first report indicating that ATF3 promotes spontaneous metastasis and is consistent with the results of an earlier report where ATF3 was found to augment metastasis of murine melanoma cells when the cells were injected i.v. (17). Because we have shown previously that Drg-1 significantly suppressed lung metastases of the highly metastatic Dunning rat prostate cancer cells (AT6.1) and because AT2.1 cells are from the same family as AT6.1 but have low metastatic ability, the results of the animal experiment presented in this report strongly argue for the notion that Drg-1 suppresses the metastatic ability of tumor cells by inhibiting the expression of the *ATF3* gene. Considering the proinvasive activity of the *ATF3* gene noted by us and others, it can be speculated that ATF3 promotes metastasis by augmenting invasion of the cells through the extracellular matrix and/or extravasation of tumor cells at the secondary site, although the cellular and molecular details of this process remain to be understood.

Taken together, we propose a molecular mechanism of action of the metastasis suppressor gene *Drg-1*, where Drg-1 down-regulates the expression of the *ATF3* gene leading to suppression of invasion and metastasis. For metastatic cancer, Drg-1 is significantly down-regulated, which in turn promotes metastatic dissemination of cancer cells, at least in part, by concomitant up-regulation of the *ATF3* gene. Further understanding of the components of this pathway should provide crucial information toward effective therapeutic intervention of metastatic cancer.

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The Tumor Metastasis Suppressor Gene Drg-1 in Cancer Progression and metastasis

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ABSTRACT

The process of tumor metastasis is negatively regulated by metastasis suppressor genes and understanding the mechanism of action of these genes provides critical insight into the complex process of metastasis. This chapter is devoted to a recently discovered metastasis suppressor gene, Drg-1. Here we summarize the work from the laboratory of ours and others, providing evidence for metastasis suppression by Drg-1, describing the clinical relevance of this gene, and the current understanding of regulation and function of this gene in the context of tumor metastasis.

INTRODUCTION

The most important aspect of cancer, from the medical point of view, is metastasis which almost invariably is the ultimate cause of death from any type of cancer. Metastasis refers to the dissemination and establishment of tumor cells from the site of origin to a distant site that involves a complex multi-step process. Following primary tumor formation, a population of tumor cells acquires invasive phenotype that results in the loss of cell-cell adhesion and cell-extracellular matrix adhesion, and proteolytic degradation of the matrix. When tumor cells become further aggressive, these cells intravasate into neighboring blood vessels and disseminate through the circulation. Those cells that survive in the circulation are arrested at distant organ sites, extravasate and lodge at the secondary sites, where the cells must also proliferate and colonize for successful metastasis. Despite its obvious clinical relevance, because of the complexity of the phenomenon, metastasis remains poorly understood at the molecular and biochemical levels. Recently, there has been significant advancement in understanding several crucial aspects of this intricate biological process with the discovery of the ‘metastasis

suppressor genes', which by definition, suppresses the process of metastasis without affecting tumorigenesis. Till date, 13 such genes have been identified and these include nm23, KAI1, Kiss1, BRMS1, MKK4, RhoGD12, RKIP, Drg-1, CRSP3, SSeCK, TXNIP/ VDUP-1, Claudin-4, and RRM1[1, 2, 3]. This chapter focuses on the regulation and function of the recently discovered tumor metastasis suppressor gene Drg-1.

STRUCTURE AND EXPRESSION PATTERN OF THE Drg-1 GENE

The Drg-1 gene was originally identified as a gene strongly induced by cellular differentiation *in vitro* and hence named as Differentiation-Related-Gene-1[4]. The Drg-1 gene belongs to the 'Ndrg' family of genes, which also includes three other members, Drg-2, 3 and 4 that encode proteins highly related to Drg-1. These members vary in the pattern of tissue-specific expression and possibly in their functions [5, 6]. Drg-1 is almost identical to the human RTP, cap43 and rit42 genes, and is homologous to the mouse genes TDD5, Ndr1 and the rat gene Bdm1 [7, 8, 9]. The cap43 and RTP genes have the same predicted amino acid sequences, although there are a few differences in the 3' untranslated region, and there is a single amino acid difference from Drg-1 [10]. In Drg-1, isoleucine is changed to threonine due to T-to-C transition, but all other Drg-1 homologous genes including the mouse genes have isoleucine at this position. TDD5 has the same amino terminal part of the protein, however, there is a significant difference in the COOH-terminus [10]. In addition to the mammalian homologues mentioned above, genes homologous to Drg-1 exists in a wide variety of organisms, such as zebrafish, fruitfly, nematode, sunflower, and *Arabidopsis* [11]. Thus Drg-1 is highly conserved across species, suggesting its role in important cellular processes.

The Drg-1 gene has been mapped to human chromosome 8q24.2 [12]. Drg-1 mRNA is detected in tissues of most of the organ systems, including the digestive tract, immunological, reproductive and urinary systems [13]. Gene expression study indicated significant variation of expression level of the Drg-1 gene among different organs, and the expression has been found to be particularly high in prostate, ovary, intestine and kidney [13]. The Drg-1 gene encodes a 43kD cytoplasmic protein that has several noticeable features, however, the biochemical function of the protein is yet largely unknown. Amino acid sequence of the Drg-1 protein reveals 3 serine phosphorylation sites, 5 calmodulin kinase 2 phosphorylation sites, 5 myristoylation sites, 3 PKC phosphorylation sites, 1 tyrosine phosphorylation site, 1 thioesterase site and a phophopantotheine attachment site. It has been shown that Protein kinase A and calmodulin kinase2 are indeed involved in the phosphorylation of this protein *in vitro* [14, 15]. At the C terminal end of the Drg-1 protein, there are 3 tandem repeats of the amino acids G-T-R-S-R-S-F-T H-T-S. Murray *et al*. recently demonstrated that the C-terminal stretch of the Drg-1 protein serves as a substrate for phosphorylation by serum- and glucocorticoid-induced kinase 1 (SGK1) which then primes it for phosphorylation by glycogen synthase kinase 3 (GSK3) [16, 17]. In addition, based on potentiometric and spectroscopic studies, Zoroddu *et al.* have proposed that this stretch may be important for Nickel binding [18]. The Drg-1 protein also contains a prominent beta-hydrolase fold characterized by at least 5 parallel beta strands, a catalytic triad in a specific order (nucleophile-acid-histidine), and a nucleophilic elbow. However, using a Bayesian computational algorithm, Shaw *et al.* have found that all of the residues that could impart hydrolytic functionality have been eliminated in the Drg-1 class of proteins, although the overall structure of the a/b hydrolase fold has been preserved [19]. Studies are underway in the

laboratories of ours and others to understand the exact biochemical function of this protein and its physiological relevance.

CANCER CONNECTIONS: EXPRESSION OF THE Drg-1 GENE IN HUMAN TUMOR TISSUES

Originally aiming at identifying genes involved in differentiation, van Belzen *et al.* utilized a colon carcinoma cell line that could be induced to differentiate *in vitro* and by using a modified differential display approach they identified Drg-1 as a novel gene strongly induced during differentiation [4]. Loss of differentiation is one of the salient features of tumor cells and tumor progression is often characterized by downregulation of differentiation related genes. In line with this idea, the differentiation-related gene Drg-1 has been found to be downregulated in several types of cancers, including prostate, breast, colon and pancreatic carcinoma [20, 21, 22, 23]. As shown in Fig.1(a), in the tissue specimens from both prostate and breast cancer cases, Drg-1 was found to be highly expressed in the epithelial cells of normal glands and ducts, and the basal cell layers also showed high level of Drg-1, where the protein was localized mostly in the cytoplasm. The stroma did not have any detectable level of Drg-1 expression, but the endothelial cells and nerve bundles frequently expressed Drg-1. The Drg-1 protein was detected consistently in all cases of normal prostate and breast tissue, as well as PIN (Prostatic Intraepithelial Neoplasia) and BPH (Benign Prostatic Hyperplasia), while the Drg-1 expression was significantly reduced in the tumor cells of nearly 47% prostate cancer and 30% breast cancer patients [20, 21]. In the case of prostate cancer, when the patients were subdivided into two groups, those with Gleason score lower than or equal to 7 and those with a Gleason score more than 7, the reduction in Drg-1 expression correlated significantly with the Gleason grade

(P=0.015) (Fig.1b). A study by Caruso *et al.* also found a similar trend of downregulation of Drg-1 expression in prostate cancer, and interestingly, they also observed a significant correlation between Drg-1 expression pattern and ethnic origin of the patients [24]. In our study population, Drg-1 expression had an overall significant inverse correlation with the degree of differentiation ($P < 0.001$) (Fig. 1b). However, one-way ANOVA test with Tukey's W procedure indicated that the down regulation of Drg-1 is not significant between well and moderately differentiated tumors though it is highly significant between moderate and poorly differentiated tumors. These results are in agreement with the idea of this gene being up-regulated by induction of cellular differentiation *in vitro* and also suggest a possibility that Drg-1 suppression may be more important in the late stage of tumor progression. Indeed, in both prostate and breast cancer, we observed a significant level of differential expression of Drg-1 between the patients with organ-confined disease and those with metastasis to lymph node or bone (Fig.1b, ref.21). For instance, in the case of prostate cancer, while 28 cases (70%) were positive for Drg-1 out of 40 localized prostate cancer cases, only 5 (25%) were positive for Drg-1 expression out of each of the 20 and 19 cases with lymph node and bone metastasis. Thus, the negative correlation of Drg-1 with metastatic spread to the lymph node and the bone is highly significant ($P= 0.003$ and 0.006, respectively), and in fact, is much stronger than the positive correlation with Gleason scores. Similarly, in the case of breast cancer, while 89.7% patients were positive for Drg-1 expression out of 29 cases with localized disease, only 60.7% were positive for Drg-1 expression among 56 patients with metastases [21]. These results strongly suggest the negative involvement of Drg-1 in the process of invasion and metastasis in both prostate and breast cancer, which also is in good agreement with the recent observation by

Maruyama *et al.* that Drg-1 expression has a significant inverse correlation with depth of invasion in pancreatic adenocarcinoma patients [23].

In addition to reduction in expression of the Drg-1 gene in tumor tissue, recent studies have also indicated the prognostic importance of this gene. In the case of prostate cancer, patients with Drg-1 positive expression had significantly more favorable prognosis than those with reduced expression of the gene (P=0.002, log rank test) (Fig.1c). Consistently, in a group of 85 breast cancer cases, patients with Drg-1 positive expression had significantly better prognosis than those with reduced expression of the gene (P=0.002) (Fig 1c). Recently, Maruyama *et al.* have also observed that reduced expression of the Drg-1 (cap43) gene is significantly (P=0.0062) associated with poor overall survival rate in pancreatic ductal adenocarcinoma [23]. Furthermore, in multivariate Cox regression analysis involving the Drg-1 expression status in breast cancer, primary tumor size and degree of metastasis, we found Drg-1 to be an independent and statistically significant prognostic factor. The odds ratio for Drg-1 was 2.4 (95%CI 1.03-5.76, P=0.043), implying that the death risk of breast cancer patients with reduced Drg-1 expression within a specific time was 2.4 times higher than the risk of patients to die within the same time course with Drg-1 positivity [21]. Thus, the reduced expression of Drg-1 can be a strong predicator of lymph node and bone metastasis and, in turn, of survival in breast cancer patients. In Cox regression analysis in univariate mode, the Drg-1 gene expression in prostate cancer also had a significant predictive value (P=0.0256), although it was less predictive than lymph node or bone metastasis (P<0.001) [20]. Taken together, these data underscore the clinical relevance of the Drg-1 gene in advancement of human cancer.

EVIDENCE OF METASTASIS SUPPRESSION BY Drg-1

The significant inverse correlation of Drg-1 expression with the extent of metastasis at the clinical level suggests a potential role of this gene in the process of tumor metastasis. However, the definitive proof of such action of the gene can only be obtained from experiments in animal model. AT6.1 is a dunning rat prostate cancer cell line and rapidly grows into primary tumor in SCID mice when subcutaneously injected followed by high incidence of lung metastasis. Therefore, it provides a useful model for studying spontaneous metastasis *in vivo*. We transfected the mammalian expression plasmid of Drg-1 into AT6.1 cells, and selected several permanent clones with strong Drg-1 expression as shown in Fig 2a. Each of these clones was individually injected subcutaneously into the dorsal flanks of SCID mice and monitored for tumor formation and growth rate of the tumor. Five weeks after the inoculation of the cells, the mice were sacrificed and the number of metastatic lesions on their lungs was grossly counted. We found that all the clones formed primary tumors in the animals with similar growth rates during the 5-week period, suggesting that Drg-1 does not have an effect on tumorigenesis and primary tumor growth rate. On the other hand, as shown in Fig.2, the clones that were positive for Drg-1 expression showed a significantly lower incidence of lung metastases compared with the vector-transfected cell line and the clone (#12) negative for Drg-1 expression. These results strongly suggest that Drg-1 has the ability to suppress the metastatic dissemination of prostate cancer cells without affecting tumorigenicity *in vivo*.

Similar metastasis suppressor effect of Drg-1 was also observed in colon carcinoma cells by Guan *et al.* [22]. When three Drg1-transfected clones and two empty vector clones were injected into the spleen of athymic nude mice, the tumor burdens of the splenic primary tumors were very similar between the transfected and control groups. However, 75% mice developed liver metastases in the empty vector control groups whereas only 23% had liver metastasis in the

Drg-1-transfected group, strongly suggesting that Drg-1 may function as a suppressor of colon cancer metastasis without altering the ability of the cells to form primary tumor [22]. Furthermore, in consistence with the metstasis suppressor action of the Drg-1 gene, Yoshizumi and colleagues recently demonstrated that treatment of colon cancer cells with a PPAR(Peroxisome proliferator-activated receptor)-gamma ligand and differentiation-inducing agent, thiazolidinedione (TZD), completely inhibited lymph node and lung metastases in a xenograft animal model, and this was associated with a marked increase in Drg-1 expression [25]. On the other hand, Kurdistani *et al.* demonstrated that when EJ bladder cancer cell line overexpressing the Drg-1 gene was injected into nude mice, primary tumor mass was significantly reduced compared to parental cell line [12]. In line with such finding, Stein et al. recently observed that Drg-1 is a crucial factor in p53- mediated apoptosis in DLD-1 colon cancer cells [26]. These results result suggests that, depending on the cellular context, Drg-1 is also capable of suppressing primary tumor growth, although the factor that contributes to such dichotomous function of the Drg-1 gene is yet to be understood. It should be noted that Okuda *et al.* recently generated Drg-1 knock-out mouse which does not exhibit any spontaneous tumor phenotype, consistent with the notion that Drg-1 acts as a metastasis suppressor gene without affecting primary tumorigenesis *in vivo* [27]. Therefore, it will be of great interest to cross the Drg-1 knock-out mouse with a spontaneous metastasis mouse model and assess for potential suppression of metastasis.

REGULATION OF THE Drg-1 GENE IN TUMOR CELL

It is evident from both animal studies and clinical studies that Drg-1 acts as a metastasis suppressor gene, and therefore, it is of paramount interest to understand how this gene is down-

regulated in tumor cells so that this information may lead to the design of effective therapeutic strategy to target metastatic cells in cancer patients. Since deletion or loss of human chromosome 8q24.3, where Drg-1 is localized, is not a common event in human cancers, it is plausible that down-regulation of the Drg-1 gene occurs at the transcriptional or translational level. Indeed, RT-PCR analysis on breast cancer samples from patients with metastatic disease revealed that there is a significant reduction of Drg-1 mRNA in the tumor cells in 75% cases compared to the normal counterparts, suggesting that the reduction of the expression of the Drg-1 gene in cancer cells is, for the most part, at the RNA level [21].

Epigenetic regulation of the Drg-1 gene

One notable mechanism of gene regulation at the RNA level that has been observed in different types of human cancers is aberrant methylation of cytosines located 5' to guanosines (CpG) in the promoter region of tumor suppressor and metastasis suppressor genes. Scanning of the 5' upstream region of the Drg-1 gene revealed two prominent CpG, islands suggesting that DNA methylation may contribute to the regulation of this gene. Indeed, treatment of a panel of human breast carcinoma cell lines with the DNA methylation inhibitor, 5-Azacytidine indicated that demethylation resulted in a significant increase in the expression of Drg-1 at both mRNA and protein levels [21]. Hypermethylation has been shown to down-regulate Drg-1 expression in colon cancer cells as well [22]. These results strongly suggest that Drg-1 expression at the transcriptional level is controlled, at least in part, by hypermethylation of CpG islands and that inhibition of methylation is capable of restoring the expression of the Drg-1 gene. Drg-1 expression is also regulated by histone deacetylation, since Drg-1 mRNA was found to be markedly upregulated by treatment with histone deacetylase inhibitors in colon and nasopharyngeal cancer cell lines [22, 28].

Regulation of Drg-1 by multiple of factors / pathways

In addition to epigenetic mechanisms, Drg-1 is also controlled by multiple factors and is responsive to various stimuli *in vitro* (Fig. 3). The tumor suppressor gene p53 has been shown to regulate the expression of Drg-1 albeit in a cell type specific manner. Kurdistani *et al*. have demonstrated that the tumor suppressor gene p53 is able to induce the expression of Drg-1 in p53-null bladder cancer cell line and fibrosarcoma cells, while Stein *et al.* recently found that p53 induced expression of the Drg-1 gene in non-metastatic colon cancer cell lines DLD-1 and HCT-1 but not in the metastatic lung cancer cell line H1299 [12, 26]. On the other hand, the tumor suppressor gene von Hippel Lindau has been shown to transcriptionally downregulate the expression of the Drg-1 gene in a renal cancer cell line, although such regulation is yet to be clarified *in vivo* [29]. In a separate study, utilizing N-myc deficient mouse embryos, Shimono *et al.* suggested that Drg-1 expression was down-modulated by N-myc, and indeed, N-Myc and Max were found to repress the promoter activity of the Drg-1 gene [8]. They also observed that the Drg-1 promoter was equally repressed by c-Myc and max, suggesting that if N-Myc or c-myc activity is augmented during malignant transformation of the cells, then Drg-1 expression would be repressed [8].

Results of several *in vitro* studies have also indicated that Drg-1 is a stress responsive gene and various chemical agents including homocysteine, mercaptoethanol, tunicamycin, lysophosphatidylcholine, and synthetic retinoids have been shown to induce the expression of this gene in cultured cells [5, 30, 31]. In addition, Richardson *et al.* found that treatment of cells with Fe-chelators specifically upregulated the expression of the Drg-1 gene [32]. Since Fe is a critical factor in cell proliferation, this result suggests that Drg-1 is a novel link between iron metabolism and control of cell proliferation. Furthermore, Drg-1 has been found to be

upregulated in human carcinoma cells following treatment with nickel compounds via elevation of free intracellular Ca^{2+} levels [10]. Consistent with this finding, induction of Drg-1 expression by nickel, calcium ionophore or okadaic acid can be blocked by bis-(O-aminophenoxy)-ethane NNNN tetraacetic acid tetra-(acetoxymethyl)-ester [10]. It has been also demonstrated that acute exposure to nickel results in accumulation of hypoxia-inducible transcription factor (HIF)-1, which strongly activates hypoxia-inducible genes, including Drg-1 [33].

In the case of prostate cancer, it should be noted that the Drg-1 gene was previously shown to be upregulated by androgen in LnCap prostate cancer cell line [34]. On the contrary, Lin *et al.* observed that the rat homologue of Drg-1, TDD5, was repressed by testosterone and dihydrotestosterone [7]. They further suggested that TDD5 is an early responsive androgen target gene, since their animal studies showed that TDD5 mRNA levels were repressed within 8 hours after dihydrotestosterone administration [7]. Thus, regulation of Drg-1 expression by androgen remains controversial. In fact, we did not observe any significant correlation between expression of Drg-1 and androgen receptor in immunohistochemical analysis of clinical samples of prostate cancer, indicating that androgen signaling may not be a critical factor for regulation of Drg-1 expression *in vivo* [35].

Tumor suppressor gene PTEN upregulates the Drg-1 gene

PTEN is one of the most common targets of mutation in human cancers, with a mutation frequency approaching that of p53 [36]. In the case of human prostate cancer, deletion and /or mutations of the PTEN gene are reported in 30% of primary and 63% of metastatic tumors, placing PTEN among the most common genetic alterations in this type of cancer [37, 38]. In a microarray analysis, Unoki *et al.* recently identified Drg-1 as one of the several genes up-regulated by PTEN in two endometrial cancer cell lines [39]. In our study, introduction of

PTEN in PTEN-null prostate and breast cancer cells dramatically upregulated the endogenous level of Drg-1, while knock-down of PTEN gene significantly reduced Drg-1 expression in prostate cancer cells which strongly suggest that Drg-1 is positively regulated by PTEN at least *in vitro* [35]. This regulation of the Drg-1 gene by PTEN occurs at the transcriptional level since we observed that PTEN over-expression significantly augmented the activity of 1.5kb promoter region of the Drg-1 gene [35]. PTEN is a dual specificity phosphatase that inhibits PI3K dependent activation of Akt, and deletion or inactivation of PTEN results in constitutive Akt activation [40]. In line with such cross-talk between PTEN and PI3K, we found that treatment of prostate cancer cells with the PI3K inhibitor Ly-29400 that decreased the phospho-Akt level, also resulted in a concomitant increase in Drg-1 expression [35]. Together, the results of our *in vitro* experiments strongly implicate that PTEN transcriptionally upregulates the expression of the Drg-1 gene via an Akt-mediated pathway.

Expression of the PTEN and Drg-1 genes were also found to have a significant positive correlation in clinical setting of prostate and breast cancer, which is consistent with the notion that PTEN controls the expression of the Drg-1 gene [35]. Furthermore, we found that in univariate survival analysis, patients negative for both PTEN and Drg-1 had significantly worse prognosis than those with positive expression of either one or both markers [35]. Importantly, Cox regression analysis revealed that the combination of PTEN and Drg-1 gene expression was an independent prognostic marker in both prostate and breast cancer, and the death risk of a patient with negative expression of both markers was significantly worse than those positive for both or either PTEN and Drg-1 [35]. These data underscore the prognostic importance of combination of PTEN and Drg-1 and also point toward the clinical relevance of the PTEN-Drg-1 pathway in metastatic advanvcement of prostate and breast cancer.

The finding that the tumor suppressor gene PTEN gene upregulates the tumor metastasis suppressor gene Drg-1 has several implications especially for the biology of prostate cancer. PTEN has been shown to be frequently mutated in various types of cancers, including glioblastoma, melanoma, endometrial, breast, lung, gastric, colorectal, bladder, and head and neck cancer [36]. In most of these cases, PTEN inactivation was also found to have a significant correlation with invasiveness and metastasis [38, 41, 42]. Interestingly, recent studies using various mouse models have begun to reveal a functional involvement of PTEN in suppressing tumor metastasis. Using a series of hypomorphic PTEN mutant mice with decreasing PTEN activity, Trotman *et al.* have shown that the extent of PTEN inactivation dictates metastatic progression of prostate cancer in a dose-dependent manner [43]. In a separate study, Wang *et al*. demonstrated that mice with prostate specific bi-allelic deletion of the PTEN gene spontaneously develop PIN lesions followed by invasive adenocarcinoma, and more than 50% of the animals develop pulmonary metastasis by 29 weeks of age [44]. More direct link between PTEN and prostate cancer metastasis was demonstrated by Davies *et al.* in an orthotopic mouse model where *ex vivo* treatment of PC3 prostate cancer cells with adenoviral PTEN expression vector completely inhibited lymphnode metastases without inhibiting tumorigenicity [45]. *In vivo* treatment of pre-established PC3 tumors with adenoviral PTEN also markedly diminished lymphnode metastasis formation without causing significant regression of local tumor [45]. These results are in good agreement with the previous observation that reintroduction of the human 10q23-25 region into highly metastatic rat prostate cancer cells significantly suppressed metastasis without affecting their tumorigenic potential [46]. The metastasis suppressor role of PTEN was also suggested in the case of a melanoma mouse model where overexpression of PTEN in B16F10 cells inhibited

experimental pulmonary metastasis [47]. Taken together, the results of these animal experiments implicate a critical role of the PTEN gene in tumor metastasis. Our finding, that PTEN upregulates the expression of the Drg-1 gene, strongly suggests that metastasis suppressor function of PTEN is at least in part mediated by Drg-1.

MECHANISM OF METASTASIS SUPPRESSION BY Drg-1

Results from animal experiments as well as clinical studies provide compelling evidence supporting the notion that the Drg-1 gene is a novel tumor metastasis suppressor, and that the status of the expression of this gene may serve as a diagnostic and prognostic marker. The next most intriguing question that needs to be addressed is how the Drg-1 gene exerts its metastasis suppressor function. Metastasis is a complex process involving a cascade of events and the steps that are affected by Drg-1 are largely unknown, but studies from different laboratories have begun to shed light on the functional role of this gene in various types of cancer. Drg-1 has been found to drammatically suppress the invasive ability of prostate and breast cancer cells in the Matrigel assay *in vitro* [20, 21]. Drg-1 however did not significantly affect the migratory property of the tumor cells in this assay. Notably in separate studies from different laboratories, Drg-1 has also been shown to inhibit invasiveness of colon cancer cells, pancreatic cancer cells and hepatocellular carcinoma cells [22, 23, 48]. These data strongly suggest that Drg-1 suppresses the invasive ability of aggressive cancer cells *in vitro*, which is consistent with the results of immunohistochemical analysis of clinical specimens by us and other groups [20, 21, 23]. In this context, it should be noted that Drg-1 has been found to be up-regulated by the tumor suppressor gene PTEN which is also known to be able to down-regulate metastasis-related genes such as MMP-1, 2 and 13 [49, 50, 51]. Therefore, it is tempting to speculate that Drg-1

may be involved in down regulation of these protease genes by PTEN, which may at least partly account for the metastasis suppressor function of the Drg-1 gene. The effect of Drg-1 on tumor cell proliferation, however, remains elusive. In the case of prostate cancer, it was found that cells stably expressing Drg-1 did not significantly differ from the vector-transfected control cells in terms of growth rate in two-or three-dimensions or any morphological features [20]. Consistently, in separate studies, the rate of proliferation of pancreatic adenocarcinoma and metastatic colon carcinoma (SW620) cells was found to remain unaltered by Drg-1 expression [23, 22]. Interestingly, however, Stein *et al.* recently observed that Drg-1 inhibits proliferation of metastatic lung cancer cells (H1299) but does not affect the growth of non-metastatic colon cancer cells (DLD-1) [26]. In addition, growth-inhibitory property of Drg-1 has been observed in breast (MCF7) and bladder (EJ) cancer cells, and Drg-1 has been also shown to acts as an inhibitor of polyploidy in p53-null tumor cells [12, 52]. Drg-1 may therefore affect cell proliferation albeit in a cell-type-specific and/or context-dependent manner. In terms of metastasis suppression, it is plausible that Drg-1 affects the growth of tumor cells at the secondary site although the factor(s) that trigger the growth-inhibitory property of this gene remains to be understood.

To gain mechanistic insight into the functional role of the Drg-1 gene as a metastasis suppressor, several approaches are underway in our laboratory. Recently, using microarray gene expression analysis technique, we have found that Drg-1 significantly suppressed expression of the ATF3 gene which was previously known as a stress inducible transcription factor [manuscript under preparation]. The ATF3 gene, also known as LRF-1 (Liver regeneration factor-1), belongs to the ATF/CREB family, and as a homodimer acts as a transcriptional repressor on various promoters while it functions as suppressor when it forms a heterodimer [53].

We have also observed that ATF3, when stably transfected into prostate carcinoma cells, significantly promotes invasiveness of the cells. More importantly, ATF3 overexpression significantly enhanced the spontaneous pulmonary metastasis of the rat prostate carcinoma cells AT2.1, which otherwise have a low metastatic potential. At the clinical level also, we have observed a significant negative correlation between Drg-1 and ATF3 expression in the case of prostate cancer. These findings strongly support our notion that Drg-1 suppresses tumor metastasis by inhibiting the function of the ATF3 gene. Consistent with our observation of the metastasis-promoting role of ATF3, Ishiguro *et al.* have previously observed that ATF3 enhanced experimental metastasis of murine melanoma cells and that antisense blocking of the ATF3 mRNA inhibited cell migration and invasion [54, 55].

CAN CANCER METASTASIS BE VIEWED AS A STEM CELL DISEASE?

Cancer stem cells have recently been identified in a number of solid tumors and have been proposed to be the critical cell population for initiation and propagation of cancer [56]. Interestingly, Karhadkar *et al.* have found that blockade of the sonic hedgehog (Shh) pathway by the specific pathway inhibitor cyclopamine led to concomitant upregulation of the Drg-1 gene in metastatic prostate cancer cell lines, PC3 and Du-145, while benign prostate epithelial cells exhibited high basal level of Drg-1 that remained unchanged by cyclopamine treatment [57]. While the Shh pathway plays indispensable role in embryonic pattern formation, it is also essential for maintenance of the pool of adult stem cells in various organs where the misappropriate activation of the pathway leads to tumorigenesis [56]. In the case of human prostate cancer, Shh pathway activity has been found to be dramatically augmented in the cells that have metastasized compared to those that are localized [57]. It is therefore plausible that

Shh promotes metastasis, at least in part, by inhibiting the expression of the metastasis suppressor gene Drg-1 and that Drg-1 plays a crucial role in blocking metastatic dissemination of tumor stem cells.

CONCLUSION

Drg-1 is a recently discovered metastasis suppressor gene which is at the center of wide array of important regulatory factors. Based on our experimental findings as well as current literature, we propose that several factors including the tumor suppressor gene PTEN upregulate the expression of the Drg-1 gene, which in turn suppresses ATF3, thereby inhibiting metastatic colonization at the secondary site. We have just begun to understand the molecular mechanism of action of this gene as a metastasis suppressor, and there are several crucial questions that remain to be answered. We are making an effort to understand what are the interactors of the Drg-1 protein, and how such interaction(s) modulate the activity of Drg-1. It will be also interesting to unravel any cross-talk that may exist between Drg-1 and other tumor metastasis suppressors and perceive the network of action of the metastasis suppressor genes in tumor cells.

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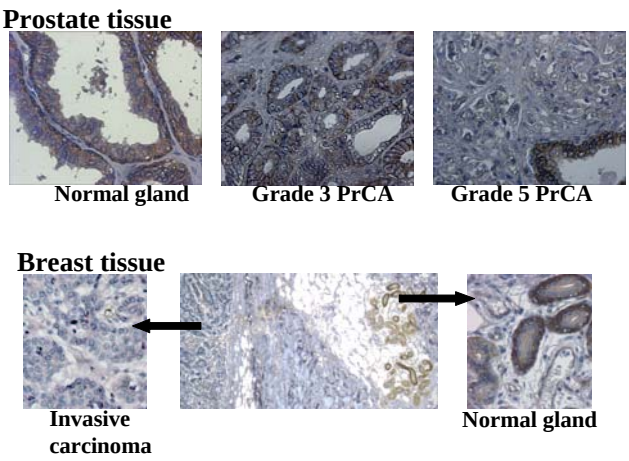
FIGURE LEGENDS

Fig. 1. Immunohistochemical analysis of Drg-1 in human prostate and breast cancer. (a) Using anti-Drg-1 antibody, immunohistochemistry was performed on paraffin tissue sections from prostate and breast cancer patients of various grades. (b) Association of Drg-1 with other clinical parameters in prostate cancer. In each case, chi-squared test was performed to test the significance of association. * indicates statistically significant correlation ($P<0.05$). (c) Drg-1 expression is correlated with overall survival rate. Overall survival rate over a period of 5 years was measured in patients with prostate and breast cancer, in relation to Drg-1 expression. The solid and dotted line indicate patients with positive and reduced expression of Drg-1, respectively. P value was determined by log rank test.

Fig. 2. Drg-1 suppresses spontaneous lung metastasis without affecting growth of primary tumor. (a) The parental cell line AT6.1, AT6.1 cells transfected with empty vector (vector only), and Drg-1 positive (#4, #7, #8, #10) and negative (# 12) clones were injected subcutaneously into SCID mice (5 mice per group). After 4 weeks, the mice were sacrificed and the lungs were removed. The tumor nodules on the lungs were counted macroscopically. The lungs from two mice from each group are shown as examples. (b) The table summarises the data from the animal experiment described above.

Fig. 3 Proposed regulation and mechanism of action of Drg-1.

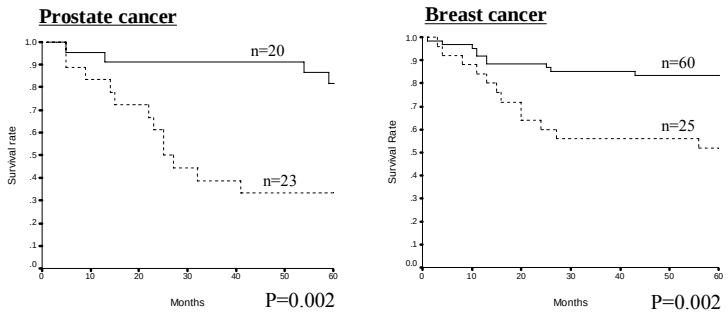
(a)



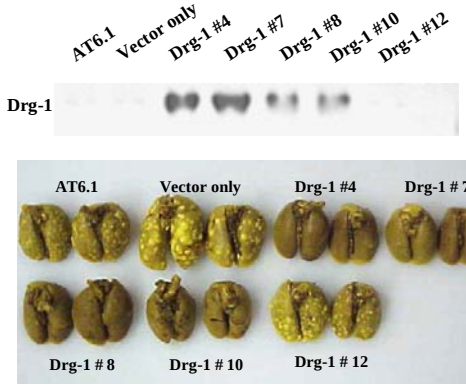
(b)

	All (62)	Drg-1 expression positive (34)	reduced (28)	P value
Gleason grade				
≤ 7	**38**	**26**	**12**	
> 7	**24**	**8**	**16**	**0.015***
Differentiation				
Well	**16**	**14**	**2**	
Moderate	**19**	**14**	**5**	
Poor	**27**	**6**	**21**	**<0.001***
Metastasis status				
Organ confined	**40**	**28**	**12**	
Lymph node metastasis	**20**	**5**	**15**	**0.003***
Bone metastasis	**19**	**5**	**14**	**0.006***

(c)

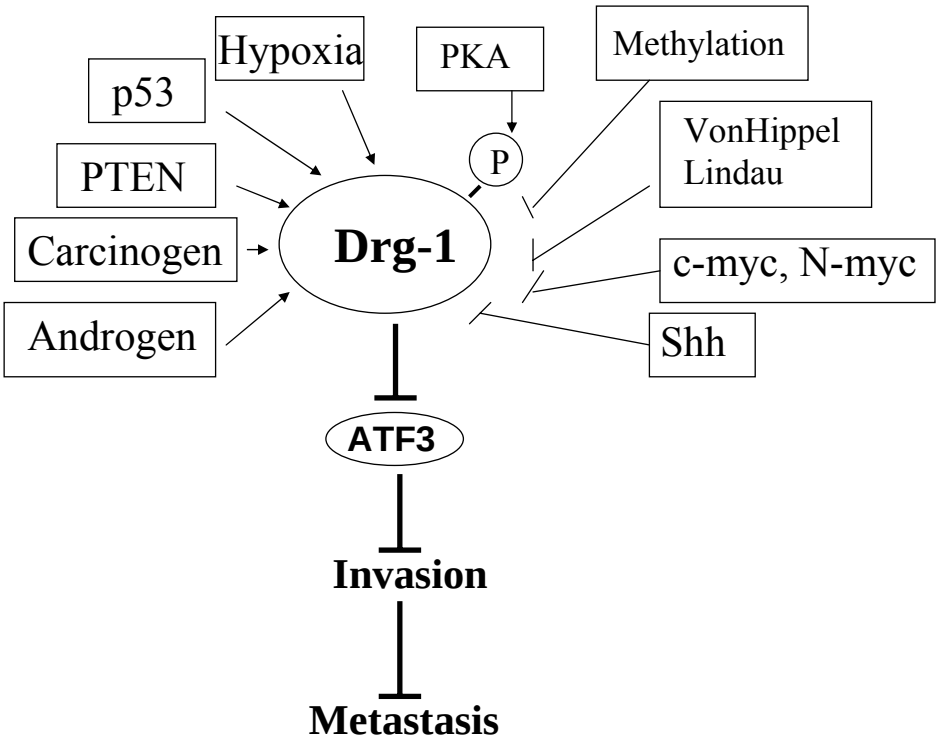


(a)



(b)

Cell line	Drg-1[a]	Tumor incidence[b]	In vivo doubling time	Lung metastases Mean+/-SE[d]	Median[d]	(Range)[e]	P value
AT6.1	-	5/5	3.4 +/- 0.3	153.7 +/- 22.0	150	(116-195)	
Vector only	-	5/5	3.3 +/- 0.4	134.5 +/- 22.8	110	(78-240)	0.7
Drg-1 #4	+	5/5	3.4 +/- 0.3	13.7 +/- 6.6	10	(3-32)	<0.001*
Drg-1 #7	+	5/5	3.0 +/- 0.2	5.8 +/- 2.5	2	(2-14)	<0.001*
Drg-1 #8	+	5/5	2.9 +/- 0.4	11.4 +/- 5.5	9	(0-11)	<0.001*
Drg-1 #10	+	5/5	3.2 +/- 0.5	1.0 +/- 0.5	1	(0-3)	<0.001*
Drg-1 #12	-	5/5	3.4 +/- 0.4	176 +/- 33.1	180	(80-280)	0.7


Hypoxia
PKA
Methylation
p53
VonHippel
Lindau
P
PTEN
Drg-1
Carcinogen
c-myc, N-myc
Androgen
Shh
ATF3
Invasion
Metastasis

Abstract: AACR 2006

Expression of RhoC correlates with metastatic disease and survival of prostate cancer patients

Iiizumi M, Bandyopadhyay S, Hirota, S, Hosobe, S, Tsukada T, Miura K, Saito K, Watabe M, Furuta E, Zhan R, Pai S, Mohinta S and Watabe K.

RhoC is a member of the RAS superfamily of small GTP-binding proteins. Rho GTPases have previously been shown to be involved in controlling cytoskeletal reorganization through MAP kinase pathway during cell migration and invasion, and they are considered to play key roles in tumor progression. The expression of RhoC appears to be highly up-regulated in various types of cancers including, breast, melanoma, pancreatic and lung. Moreover, inhibition of the expression of this gene was shown to significantly reduce the invasiveness of tumor cells in vitro, suggesting the role of RhoC in tumor metastases, although the molecular mechanism of RhoC action is yet to be examined. In order to understand the functional roles of RhoC in tumor metastases in prostate cancer, we performed immunohistochemical analysis on tumor specimens from 56 prostate cancer patients. RhoC was generally undetectable or only weakly expressed in normal epithelia and stroma cells, however, it was found to be significantly over-expressed in the cytoplasm of high grade tumors. The patients with high Gleason score (>7) had a tendency of higher expression of RhoC, although it was statistically not significant. However, the status of both lymphnode and distant metastatis have significant positive correlation with the RhoC expression ($P<0.03$). Interestingly, the RhoC expression also showed significant inverse correlation to that of Drg-1 which is a recently identified metastases suppressor gene. Further analysis of five year survival data revealed that the RhoC expression was significantly correlated to patient survival ($P<0.02$), and that Cox regression analysis indicated RhoC as an independent marker for the prediction of patient outcome. We also examined the effect of RhoC over-expression in prostate tumor cell lines and found that RhoC significantly suppressed both invasion and motility in vitro. Furthermore, results of western blot analysis of these RhoC over-expressed cells indicated that RhoC significantly altered the status of the phosphorylation of the Akt protein. Taken together, our results indicate that RhoC is involved in the process of tumor metastases in prostate cancer by augmenting cell invasiveness through a modulation of the Akt pathway, and that RhoC may serve as a useful prognostic marker.

Abstract: AACR 2006

Mechanism of apoptosis induced by the inhibition of Fatty Acid Synthase in breast cancer cells

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Department of Medical Microbiology and Immunology, Southern Illinois University School of Medicine, Springfield, Illinois, USA[1]. Akita Red Cross Hospital, Akita city, Japan[2].

Fatty acid synthase (FAS), a key enzyme of the fatty acid biosynthetic pathway, has been found to be overexpressed in a wide range of epithelial tumors including breast cancer. Inhibition of FAS leads to apoptosis of breast cancer cells in culture and also results in decreased tumor size in mouse models. However, the molecular mechanism by which inhibition of FAS induces apoptosis in tumor cells remains largely unknown. To understand the apoptotic pathway resulting from FAS inhibition, we utilized an apoptosis and cell-cycle-specific microarray and found that the pro-apoptotic genes, BNIP3, TRAIL and DAP kinase 2 were significantly upregulated upon direct inhibition of FAS by siRNA. The result was further confirmed by real-time RT-PCR and western blot analyses. We also explored a possibility that cell death caused by inhibition of FAS is mediated by sphingolipid ceramide, and found that knock-down of FAS expression by siRNA indeed significantly increased ceramide level in the breast tumor cells. Furthermore, treatment of tumor cells with FAS-siRNA in the presence of a ceramide synthase inhibitor abrogated the upregulation of BNIIP3 and inhibited apoptosis, indicating that FAS-siRNA induces apoptosis via accumulation of ceramide followed by upregulation of the BNIP3 gene. Consistently, we also observed a significant inverse correlation in the expression of FAS and BNIP3 in clinical samples of human breast cancer. Therefore, collectively, our results indicate that inhibition of FAS expression in breast cancer cells leads to accumulation of ceramide and induction of the pro-apoptotic genes, BNIP3, TRAIL and DAP kinase2, resulting in apoptosis.

Schedule

Opening Remarks, 8:15 A.M.
Marsha Rosner, Ph.D.
Michelle LeBeau, Ph.D.

Session I, 8:30 A.M. - 11:15 A.M.
Session Chair: Carrie Rinker-Schaffer, Ph.D.
8:30 - 9:15, Danny Welch, Ph.D.
9:15 - 10:00, Patricia Steeg, Ph.D.
10:00 - 10:30, *Break*
10:30 - 11:15, John Isaacs, Ph.D.

Session II, 11:15 A.M. - 3:30 P.M.
Session Chair: Kay Macleod, Ph.D. 11:15 - Noon, Ann Chambers, Ph.D.
Noon - 1:30, *Lunch*
1:30 - 2:15, Chand Khanna, DVM, Ph.D.
2:15 - 3:00, John Condeelis, Ph.D.

3:00 - 3:30 P.M., *Break*

Session III, 3:30 - 5:00 P.M.
Session Chair: Geoffrey Greene, Ph.D.
3:30 - 4:15, David Lyden, M.D., Ph.D.
4:15 - 5:00, Kounosuke Watabe, Ph.D.

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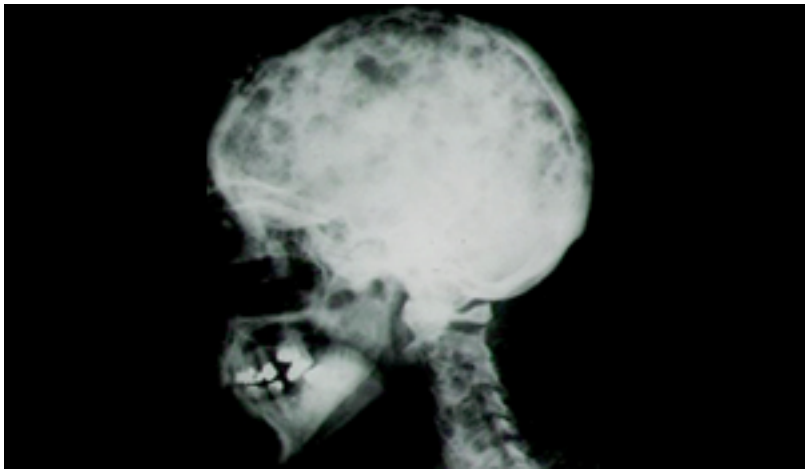

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